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(54) Title: *RIN2*, A NOVEL INHIBITOR OF RAS–MEDIATED SIGNALING

Examples of normal responses regulated by Ras-dependent signaling	Settings in which it is desirable to decrease such responses	Settings in which it is desirable to increase such responses
Mast cell mediator production	Asthma, hay fever, atopic dermatitis	Immunity to pathogens
T cell function	Graft rejection, T cell dependent diseases (certain autoimmune diseases, contact dermatitis, graft-versus-host disease)	HIV infection, certain other infections, immunosupressions associated with cancer therapies
Cell proliferation	Cancer, proliferation of vascular wall cells in arteriosclerosis	Wound healing, angiogenesis (e.g. to improve ischemia, re-epithelialization) nerve regeneration

(57) Abstract

Novel gene, *Rin2*, and encoded protein are disclosed which can inhibit the functional response induced by Ras–dependent signaling pathways are disclosed. Methods of inhibiting or enhancing Ras–dependent signaling and methods of treatment utilizing *Rin2* are also disclosed.

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RIN2, A NOVEL INHIBITOR OF RAS-MEDIATED SIGNALING

RELATED APPLICATIONS

5   The present application is a Continuation-in-Part

of U.S. Application Serial No. 08/942,819, filed October

2, 1997. The present application also claims the

benefit of U.S. Provisional Application No. 60/058,520,

filed September 11, 1997. The teachings of the prior

10 applications are incorporated herein in their entirety.

BACKGROUND OF THE INVENTION

       The Ras family of small guanine-nucleotide binding

20 proteins plays a pivotal role in many intracellular

signal transduction pathways, including those which

regulate cellular growth and differentiation and those

which contribute to cell activation (Bourne et al.,

Nature 348:125-132 (1990); Marshall, FASEB J.

25 9:1311-1318 (1995)). Moreover, many different receptors

expressed on the surface of diverse cell types can

result in the activation of signal transduction pathways

that are importantly influenced by Ras, and these

pathways in turn determine whether, and to what extent,

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these cells respond to such cell surface receptor-
dependent activation by proliferating, differentiating
(i.e., developing new functional characteristics),
and/or expressing specific functions (Figure 1).

5   Depending on the circumstances, these "down-stream"
consequences of the activation of Ras-dependent signal
transduction pathways can have either adaptive
(physiological) or maladaptive (pathological)
consequences. For example, controlled Ras-dependent
10 cellular proliferation is required for wound healing,
whereas poorly regulated Ras-dependent cellular
proliferation can result in the development of cancer
and other neoplasms. Similarly, appropriate Ras-
dependent cell secretion of histamine, serotonin,
15 cytokines and other mediators can be important for host
defense against parasites and other pathogens, whereas
the inappropriate activation of these same pathways, for
example, by a reaction to a bee-sting in patients who
are allergic to components of bee venom, can lead to
20 fatal anaphylaxis. Thus, Ras represents a major
regulator of many of the most fundamental biological
processes involved in both health and disease.

The mechanism by which Ras regulates such
processes, through interactions with other intracellular
25 molecules, is quite complex. Ras proteins are
membrane-associated proteins that cycle between an
active GTP-bound form and an inactive GDP-bound form.
As illustrated in Figure 1, evidence is accumulating for
the existence of many different classes of positive or
30 negative regulators of Ras and positive or negative Ras
signaling effectors, all of which, by definition, are
thought to interact directly with the active GTP-bound

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form of Ras to influence cellular signaling for growth,
differentiation and expression of function (Boguski and
McCormick, *Nature 366*:643-654 (1993); Marshall, *FASEB J.
9*:1311-1318 (1995); Marshall, *Curr. Opin. Cell Biol
8*:197-204 (1996)).

For example, some of the best-characterized Ras
regulators include the GTPase activating proteins (GAPs)
and guanine nucleotide exchange factors (Boguski and
McCormick, *Nature 366*:643-654 (1993)). The GAPs
represent a family of Ras-binding proteins which
stimulate the intrinsic rate of Ras GTP hydrolysis and
thus negatively regulate the Ras-induced signaling by
accelerating the conversion of active GTP-bound form of
Ras to the inactive GDP-bound form. Recent studies have
identified several GAPs specific for Ras proteins, which
include p120-Ras GAP, neurofibromin (the protein encoded
by the neurofibromatosis type 1 (NF1) gene), Gap1,
Ral-GDS, Rsbs 1, 2, and 4, Rin1, MEKK-1, and
phosphatidylinositol-3-OH kinase (PI3K) (Boguski and
McCormick, *Nature 366*:643-654 (1993)).

In contrast to Ras regulators, which function
primarily by influencing the amount of Ras which is in
the GTP-bound active, as opposed to the GDP-bound
inactive, form, Ras effectors are thought to influence
the ability of active, GTP-bound Ras to initiate
signaling (Figure 1). In the case of many Ras-
interacting proteins which can influence the intensity
of Ras-dependent signaling, it is not yet clear to what
extent they function as effectors as opposed to
regulators; such proteins can therefore be called Ras
regulators/effectors (Boguski and McCormick, *Nature
366*:643-54 (1993); Han and Colicelli, *Mol. Cell. Biol.*

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15:1318-1323 (1995); Marshall, *FASEB J.* *9*:1311-1318
(1995); Marshall, *Curr. Opin. Cell. Biol.* *8*:197-204
(1996)).

Ras is important in critical cell signaling events
5   in many cell types. For example, mast cells are
important effector cells in IgE-dependent immune
responses and allergic diseases (Galli, *New Engl. J.*
Med. *328*:257-265 (1993)), and mast cells also contribute
to host defense against parasites and bacteria
10 (Echtenacher *et al.*, *Nature* *381*:75-77 (1996); Malaviya
et al., *Nature* *381*:77-80 (1996); Galli and Wershil,
Nature *381*:21-22 (1996)). Mast cells reside in
virtually all vascularized tissues and express on their
surface the high affinity receptor for IgE (FceRI).
15 Aggregation of FceRI in mast cells by the interaction of
receptor-bound IgE with specific multivalent antigen
triggers the functional activation of mast cells, which
results in the release of a spectrum of biologically
active mediators (Ravetch and Kinet, *Ann. Rev. Immunol.*
20 *9*:457-492 (1991); Galli, *New Engl. J. Med.* *328*:257-265
(1993); Beaven and Metzger, *Immunol. Today* *14*:222-226
(1993)). Thus, mast cells activated by FceRI-dependent
mechanisms undergo degranulation, resulting in the
release of preformed mediators, such as serotonin (5-HT)
25 and/or histamine, the metabolism of arachidonic acid,
leading to the release of newly synthesized lipid
mediators, and the transcription, translation and
secretion of several cytokines (Gordon *et al.*, *Immunol.*
Today *11*:458-464 (1990); Galli, *New Engl. J. Med.*
30 *328*:257-265 (1993); Paul *et al.*, *Adv. Immunol.* *53*:1-29
(1993)).

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Knowledge of the signaling pathways which result in the FceRI-dependent secretion of mast cell mediators is increasing. In mast cells and basophils, a type of circulating leukocyte which shares many biochemical and
5 functional characteristics with mast cells (Galli, *New Engl. J. Med.* 328:257-265 (1993)), the FceRI receptor is a tetrameric complex comprised of a single 45 kDa α chain, which binds the Fc portion of IgE, a single 30 kDa β chain, and a homodimer of two 10 kDa γ chains
10 (Ravetch and Kinet, *Ann. Rev. Immunol.* 9:457-492 (1991); Beaven and Metzger, *Immunol. Today* 14:222-226 (1993)). The β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAM) which couple the receptor to the src family of protein tyrosine kinases (PTK) p56lyn
15 and p72syk (Beaven and Baumgartner, *Curr. Opin. Immunol.* 8:766-772 (1996)). The FceRI-dependent activation of these PTKs in turn activates various downstream effector pathways, including those involving PLCγ1 and the MAP kinase pathway (Beaven and Baumgartner, *Curr. Opin.*
20 *Immunol.* 8:766-772 (1996)).

Recent studies have shown that the crosslinking of FceRI in mast cells by IgE and specific antigen also results in the activation of Ras and of the associated Shc-Grb2-Sos pathway, which precedes Ras activation, and
25 that the activation of this pathway is dependent on Syk (Jabril-Cuenod et al., *J. Biol. Chem.* 271:16268-16272 (1996)). These results suggest that in FceRI-activated mast cells, as in T cells and B cells (the major types of lymphocytes responsible for cellular and humoral
30 immunity) which have been activated via the T cell receptor or B cell receptor, respectively, the Shc-Grb2-

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Sos pathway can activate the MAP kinase pathway via the activation of Ras.

An effector pathway of Ras mediated by the Raf-1/Erk-activating kinases (MEKs)/Erk-MAP kinases
5   cascade has been well-characterized in numerous systems (Treisman, *Curr. Opin. Cell Biol.* 8:205-215 (1996)), and this Ras-mediated pathway is important in the mast cell activation and mediator secretion that is induced by IgE- and antigen-dependent aggregation of FceRI in these
10   cells (Tsai et al., *Eur. J. Immunol.* 23:3286-3291 (1993); Offermanns et al., *J. Immunol.* 152:250-261 (1994); Hirasawa et al., *J. Immunol.* 154:5391-5402 (1995)). In addition, recent studies with rat RBL2H3 mast cells have shown that the FceRI induction of Ras
15   activation leads to transcriptional activation mediated by the transcription factors Elk-1 and the nuclear factor of activated T cells (NFAT) (Turner and Cantrell, *J. Exp. Med.* 185:43-53 (1997)). These Ras-dependent signaling pathways in mast cells appear to be complex.
20   Thus, activation of the Raf-1/MEK/Erk cascade appears to be necessary and sufficient for the activation of Elk-1 activity in mast cells. However, the induction of NFAT by FceRI in mast cells is also mediated in part by Rac-1, which is a putative Ras effector and a member of
25   the Rho family of GTP binding proteins (Turner and Cantrell, *J. Exp. Med.* 185:43-53 (1997)). Thus, the specific signaling pathways involved in FceRI-mediated mast cell activation are still unclear.

30   SUMMARY OF THE INVENTION

As described herein, mRNA differential display was used to identify the cDNA for a novel gene, *rin2* (also

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called Rabex-5). As also described herein, expression
of *rin2* was shown to be rapidly increased in mouse mast
cells activated through FceRI. Transfection of mouse
mast cells with an antisense *rin2* expression vector,

5   which resulted in antisense inhibition of Rin2
expression, potentiated the Ras-mediated intracellular
signaling responses that were induced by FceRI
aggregation, such as the induction of c-*fos* expression
and the activation of Erk-MAP kinase, JNK and p38 MAP

10   kinase activity. In addition, antisense inhibition of
rin2 expression in mouse mast cells significantly
enhanced the amounts of preformed mediator (serotonin)
and cytokine (IL-6) released from these cells upon
FceRI-dependent stimulation, suggesting that Rin2 exerts

15   its effects by down-regulating the functional responses
elicited by FceRI aggregation in mast cells. These
results support the role of Rin2 as a novel negative
regulator/effector of Ras in mast cells and indicate
that Rin2 interacts directly the mammalian H-Ras

20   protein.

Moreover, as also described herein, expression of
rin2 is also increased in mast cells stimulated via
activation of their major growth factor receptor (i.e.,
c-kit), in PC12 adrenal pheochromocytoma cells activated

25   via the receptor for nerve growth factor (NGF) (i.e.,
TrkA), and in T cells activated via the T cell receptor
(TCR). These findings support the role of Rin2 as a
novel general negative regulator/effector of Ras and
Ras-dependent signaling pathways in diverse cell types

30   which have been activated via distinct cell surface
receptors (Figure 2). In addition, the enhancement or
inhibition of Rin2 expression or function, in certain

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settings, resulting in the reduction or enhancement,
respectively, of Ras-dependent signaling in these
settings, may have benefit in diverse clinical problems,
some examples of which are shown in Figure 2.

5   As also described herein, the SY-A (Rin2) clone has
been used as a probe to screen a HT-29 human
adenocarcinoma cDNA library. Two overlapping cDNA
clones (HRIN2-1B1 (1.3 kb) and HRIN2-2B1 (2.9 kb)) have
been identified which are highly homologous to that of

10   the mouse Rin2 clone and which together represent the
full-length human Rin2 (Rabex-5) cDNA sequence. The
orientation of these overlapping clones is shown in
Figure 9. The nucleotide sequence of each clone is
shown in Figures 10A-B and 11A-C, respectively.

15   Accordingly, this invention pertains to isolated
Rin2 protein, which down-regulates the functional
responses elicited by Ras-dependent signaling pathways.
In one embodiment, Rin2 down-regulates the functional
responses elicited by FceRI aggregation in mast cells

20   (e.g., mammalian mast cells), including down-regulating
the amounts of preformed mediator (serotonin) and
cytokine (IL-6) released from these cells upon
FceRI-dependent stimulation. In another embodiment,
Rin2 down-regulates functional responses in mast cells

25   stimulated via activation of their major growth factor
receptor (i.e., c-kit). In another embodiment, Rin2
inhibits cell proliferation in PC12 adrenal
pheochromocytoma cells stimulated through the nerve
growth factor receptor (i.e., TrkA). In a further

30   embodiment, Rin2 inhibits functional responses in T
cells activated through the T cell receptor.

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In one embodiment, isolated Rin2 protein has the amino acid sequence of SEQ ID NO: 2. In one embodiment, the Rin2 protein is a portion of SEQ ID NO: 2 which is sufficient for Rin2 activity, e.g., binding or enzymatic

5   activity. Also included within the meaning of Rin2 protein or polypeptide are polypeptides which are less than the amino acid sequence of SEQ ID NO: 2 and have Rin2 activity, as well as proteins whose amino acid sequence is substantially similar to that of SEQ ID NO:

10   2 and have Rin2 activity.

In another embodiment, Rin2 protein is a derivative possessing substantial sequence identity with the endogenous Rin2 protein. In particular embodiments, the Rin2 protein is purified to homogeneity or is

15   substantially free of other proteins.

The invention also pertains to an isolated nucleic acid molecule or nucleotide sequence (*rin2*) which encodes Rin2 protein or polypeptide, or a portion of SEQ ID NO: 2 which is sufficient for Rin2 activity. In one

20   embodiment, the encoded Rin2 protein is a derivative possessing substantial sequence identity with the endogenous Rin2 protein. In a particular embodiment, the isolated nucleic acid molecule encodes Rin2 protein with the same amino acid sequence as endogenous Rin2

25   protein. In another embodiment, the isolated nucleic acid molecule has the same nucleotide sequence as the endogenous gene encoding Rin2 protein. In one embodiment, the isolated nucleic acid molecule has the nucleotide sequence of SEQ ID NO: 1. In another

30   embodiment, the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 12 and/or the nucleotide sequence of SEQ ID NO: 13. In a further

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embodiment, the isolated nucleic acid molecule comprises
the nucleotide sequence of SEQ ID NO: 14 and/or the
nucleotide sequence of SEQ ID NO: 15. In one embodiment
the isolated *rin2* nucleic acid molecule comprises the

5   nucleotide sequence of one or more of SEQ ID NOS: 12,
13, 14 and 15.

The invention also relates to DNA constructs
comprising the nucleic acid molecules described herein,
operatively linked to a regulatory sequence, and to

10   recombinant host cells, such as bacterial cells, fungal
cells, plant cells, insect cells and mammalian cells,
comprising the nucleic acid molecules described herein
operatively linked to a regulatory sequence. The
invention also relates to a method for preparing a Rin2

15   polypeptide, comprising culturing a recombinant host
cell described herein.

The invention also pertains to an antibody, or an
antigen-binding fragment thereof, which selectively
binds to Rin2 protein, or a portion of SEQ ID NO: 2

20   which is sufficient for Rin2 activity; in a particular
embodiment, the antibody is a monoclonal antibody. The
invention also relates to a method for assaying the
presence of Rin2 protein in a cell, e.g., in a tissue
sample, comprising contacting said cell with an antibody

25   which specifically binds to Rin2 protein.

The present invention also relates to an assay for
identifying agents which alter the activity of Rin2
protein. For example, a cell population, e.g., a mast
cell population, containing Rin2 protein can be

30   activated with a stimulus which activates at least one
Ras-dependent pathway in the cell in the presence of an

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agent to be tested, and the level of Rin2 activity can
be assessed.

The invention further relates to methods of
inhibiting the functional responses (e.g.,
5 proliferation, functional activation) elicited by
activation of Ras-dependent signaling pathways in cells,
comprising contacting the cells with an agent which
enhances or mimics the activity of Rin2 protein. The
invention also relates to a method of treating a mammal
10 in need thereof to inhibit functional responses elicited
by activation of Ras-dependent signaling pathways,
comprising administering to a mammal an agent which
enhances or mimics the activity of native Rin2 protein.

The invention further relates to methods of
15 inhibiting the functional responses elicited by FcεRI
aggregation, particularly in mast cells, comprising
contacting a mast cell population with an agent which
enhances or mimics the activity of native Rin2 protein.
The invention relates to a method of treating a mammal
20 in need thereof to inhibit the functional response
elicited by FcεRI aggregation, comprising administering
to a mammal an agent which enhances the activity of
native Rin2 protein. For example, the invention relates
to methods of inhibiting IgE and antigen-dependent
25 release of mediators from mast cells. Such methods can
be used to inhibit mediator release from mast cells and
other effector cells that express FcεRI, such as
basophils, monocytes/macrophages, dendritic cells,
Langerhans' cells and eosinophils, thereby ameliorating
30 disorders such as asthma and allergic diseases (e.g.,
hay fever and atopic eczema).

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The invention further relates to methods of
enhancing functional responses (e.g., proliferation,
functional activation) elicited by activation of Ras-
dependent signaling pathways in cells, comprising
5   contacting the cells with an agent which inhibits the
activity of Rin2 protein. The invention also relates to
a method of treating a mammal in need thereof to enhance
functional responses elicited by activation of Ras-
dependent signaling pathways, comprising administering
10 to a mammal an agent which inhibits the activity of Rin2
protein.

BRIEF DESCRIPTION OF THE DRAWINGS
        Figure 1 is a schematic illustration of the
15 pathways of Ras activation and Ras-dependent changes in
cell proliferation, differentiation and function.
        Figure 2 is an illustration of alteration (increase
or decrease) in Rin2 expression or activity and the
corresponding effect on Ras-dependent signaling. Also
20 illustrated are examples of instances in which it may be
clinically desirable to alter Rin2 expression or
activity and thereby alter, positively or negatively,
Ras-dependent signaling.
        Figure 3 is the cDNA sequence (SEQ ID NO: 1) and
25 the predicted amino acid sequence (SEQ ID NO: 2) of the
SY-A clone (Rin2). The predicted open reading frame
begins at nucleotide 60 and ends at nucleotide 1535.
        Figure 4 shows an alignment of the amino acid
sequences of Rin2 with the GTPase binding homology (GBH)
30 domains of Vps9p, Rin1, and JC265. The GBH domains of
Vps9p, Rin1, and JC265 and the GBH motifs I, II, and III
illustrated here represent those described in Burd *et*

al. (*Mol. Cell. Biol.* 16:2369-2377 (1996)). Proteins
are identified on the left, and the position of the
amino acid on each line is indicated. Identical amino
acids or conservative substitutions present in three of
5   four proteins are shaded. Conservative substitutions
for the alignment were assigned as follows: V, L, I, and
M; K, R, and H; F, Y, and W; D and E; N and Q; S and T;
and G and A.

Figure 5 is a graph of the kinetics of Erk-MAP
10 kinase activation induced by FceRI aggregation in
Cl.MC/C57.1 mast cells transfected with the control
vector (pBK-CMV) or in Cl.MC/C57.1 cells transfected
with the antisense expression vector (pBK-CMV-SYA-AS).

Figure 6 is a graph of the release of ^{3}H-serotonin
15 induced by FceRI aggregation in Cl.MC/C57.1 mast cells
transfected with the control vector (pBK-CMV) or in
Cl.MC/C57.1 cells transfected with the antisense
expression vector (pBK-CMV-SYA-AS). Release of
serotonin, assessed as percentage of specific release of
20 [^{3}H]-5HT, was measured 10 minutes after challenge. An
asterisk indicates $P < 0.05$ versus corresponding values
for cells transfected with the control pBK-CMV vector
(n=4 to 5 per point).

Figures 7A and 7B are graphs of the kinetics of IL-
25 6 release by mast cells. Figure 7A illustrates the
kinetics of IL-6 release induced by FceRI aggregation in
Cl.MC/C57.1 mast cells transfected with the control
vector (pBK-CMV) or in Cl.MC/C57.1 cells transfected
with the antisense expression vector (pBK-CMV-SYA-AS).
30 Transfected mouse mast cells sensitized with anti-DNP
IgE monoclonal antibody (mAb) for 2 hours were
challenged with DNP-HSA. Figure 7B illustrates IL-6

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release from transfected C1.MC/C57.1 mast cells 6 hours

after stimulation by different concentrations of

DNP-HSA. An asterisk indicates $P< 0.05$ versus

corresponding values for cells transfected with the

5   control pBK-CMV vector (n=4 to 5 per point).

    Figures 8A and 8B are graphs illustrating that

expression of antisense $rin2$ mRNA enhances cell

proliferation in PC12 cells, a rat adrenal

pheochromocytoma cell line. Rate of proliferation of

10 PC12 cells transfected with $rin2$ antisense expression

vector (pBK-CMV-SYA-AS), or PC12 cells transfected with

the control CMV vector (pBK-CMV), or untransfected PC12

cells, was measured by assessing [^{3}H]thymidine

incorporation in cells which had been incubated with [^{3}H]

15 thymidine for 3 hours after the cells had been cultured

overnight in normal culture medium (Figure 8A), or in

medium containing nerve growth factor (NGF) of indicated

concentrations and 0.5% FCS (+NGF; Figure 8B). An

asterisk indicates $P< 0.01$ versus corresponding values

20 for untransfected PC12 cells or PC12 cells that had been

transfected with the control pBK-CMV vector (n=4 per

point).

    Figure 9 shows the orientation of overlapping human

cDNA clones HRIN2-1B1 and HRIN2-2B1 relative to one

25 another.

    Figures 10A-B show an alignment of murine Rin2 with

the partial nucleotide sequence of human cDNA clone

HRIN2-1B1 from nucleotide 51 through nucleotide 405 (SEQ

ID NO: 12) and from nucleotide 885 through nucleotide

30 1144 (SEQ ID NO: 13). The sequence numbering is the

nucleotide number in the murine Rin2 sequence.

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Figures 11A-C show an alignment of murine Rin2 with
the partial nucleotide sequence of human cDNA clone
HRIN2-2B1 from nucleotide 532 to nucleotide 1276 (SEQ ID
NO: 14)(Figures 11A-B) and a portion of the nucleotide

5   sequence of the 3' end (SEQ ID NO: 15) (Figure 11C). In
Figures 11A-B, the sequence numbering is the nucleotide
number in the murine Rin2 sequence.

Figure 12 shows the interactions of murine Rin2
with murine H-Ras protein or yeast Ras2p protein in the

10 yeast two-hybrid assay. Different segments of the
murine Rin2 protein (shaded areas are those of greatest
sequence homology to Vps9p, JC265 and Rin1; black areas
are GBH motifs) were expressed as GAL4-activation domain
fusion proteins, whereas the murine H-Ras and yeast

15 Ras2p proteins were expressed as DNA-binding fusion
proteins. Positive interactions were assessed with both
a qualitative colony-lift β-galactosidase filter assay
using X-gal as the substrate (colony color) and a
quantitative liquid culture β-galactosidase assay using

20 chlorophenol red-b-D-galactopyranoside (CPRG) as the
substrate (relative β-gal units).

Figures 13A-B are graphs showing that *rin2*
antisense transfectants release normal levels of
preformed mediators but elevated levels of cytokines in

25 response to FcεRI aggregation. Figure 13A shows the
release of β-hexosaminidase (β-hex) or IL-6 induced by
FcεRI aggregation in C1.MC/C57.1 mast cells transfected
with the *rin2* antisense expression vector (AS) or the
control CMV vector (control). Transfectant cells

30 sensitized with anti-DNP IgE mAb for 2 hours were
challenged with DNP-HSA. Specific release of β-
hexosaminidase (background release was ≤ 6%) was

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measured 15 minutes after antigen challenge, whereas IL-
6 release was measured by ELISA assay 6 hours after
antigen challenge. The numbers over the columns
indicate the numbers of stable lines of transfectants

5   established from two independent transfections and
assayed in this experiment. An asterisk indicates
$p=0.03$ vs. control by unpaired Student's t test. Figure
13B shows the kinetics of IL-6 release induced by FcεRI
aggregation in C1.MC/C57.1 mast cells transfected with

10   the antisense (AS) or control vector. An asterisk
indicates $p<0.01$ versus corresponding values for the
control transfectant cells.

Figure 14 is a graph showing the kinetics of JNK
activation induced by FcεRI aggregation in C1.MC/C57.1

15   cells transfected with the *rin2* antisense expression or
the control vector. Findings are representative of
those obtained with 3-4 different antisense and control
transfectants.

Figure 15 is a graph showing the kinetics of p38

20   MAP kinase activation induced by FcεRI aggregation in
C1.MC/C57.1 cells transfected with the *rin2* antisense
expression vector or the control vector. Findings are
representative of those obtained with 3-4 different
antisense and control transfectants.

25

DETAILED DESCRIPTION OF THE INVENTION

In order to elucidate specific signaling pathways
involved in FcεRI-mediated mast cell activation, the
technique of mRNA differential display was used to

30   isolate cDNAs of genes that were differentially
expressed in mast cells stimulated through the FcεRI.
Work described herein identified the cDNA of a novel

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gene, *rin2*, whose expression is rapidly increased in
mammalian mast cells activated through FcεRI. The *rin2*
gene product shares significant homology with members of
a class of Ras-binding proteins, such as Rin1 and JC265

5   (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA*
    88:2913-2917 (1991); Han and Colicelli, *Mol. Cell. Biol.*
    15:1318-1323 (1995)), which have been shown to
    negatively regulate Ras-mediated signaling in
    Saccharomyces (S.) cerevisiae, and Vps9p (Burd *et al.*,

10 *Mol. Cell. Biol. 16*:2369-2377 (1996)), which is required
    for vacuolar protein sorting. As shown herein,
    inhibition of Rin2 expression potentiates both the
    intracellular signaling responses and the cellular
    secretory responses that are induced by FcεRI

15 aggregation. The work described herein supports the
    role of Rin2 as a novel negative regulator/effector of
    Ras-dependent signaling pathways in mast cells.

    The murine *rin2* gene encodes a protein of predicted
    molecular mass of 56.9 kDa which shares significant

20 homology to a class of Ras-binding proteins represented
    by JC265, Rin1 and Vps9p. Rin1 and JC265 were
    identified by virtue of their ability to suppress an
    activated Ras mutant allele in *S. cerevisiae*, indicating
    that they can negatively regulate Ras-induced signaling

25 responses (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA*
    88:2913-2917 (1991)). In addition, recent studies have
    shown that Rin1 interacts directly with the Ras protein
    in a manner similar to that observed with Raf1 and yeast
    adenylyl cyclase, which are known downstream effectors

30 of Ras (Han and Colicelli, *Mol. Cell. Biol. 15*:1318-1323
    (1995)). Thus, Rin1 binds primarily to the effector
    domain of Ras and functions as a downstream effector of

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Ras-GTP. On the other hand, the gene encoding Vps9p was
isolated in yeast by complementation of a vacuolar
protein sorting defect associated with the *vps9* mutant
(Burd *et al.*, *Mol. Cell. Biol.* 16:2369-2377 (1996)).

5   Thus, Vps9p is a 53 kDa protein required for the proper
sorting of several vacuolar proteins and may function as
part of the vacuolar protein sorting machinery in *S.*
cerevisiae.

     The highest homology shared between JC265, Rin1,

10   Vps9p, and Rin2 is found in the GTPase binding homology
(GBH) domain regions in which three putative GBH motifs
have been described (Burd *et al.*, *Mol. Cell. Biol.*
16:2369-2377 (1996)). In particular, the GBH motif II
is situated within the regions of JC265 and Rin1 that

15   have been previously found to be shared with GAP and
GAP-like proteins such as yeast IRA1 and IRA2, NF1, and
sar1 (Colicelli *et al.*, *Proc. Natl. Acad. Sci. USA*
88:2913-2917 (1991)). Furthermore, an FLP consensus
motif can be detected within the GBH motif II regions of

20   JC265, Rin1, Vps9p, and Rin2, and this FLP motif aligns
well with the FLR consensus motif (residues 901-903)
present in the block 3A region that is common to all GAP
proteins (Scheffzek *et al.*, *Nature* 384:591-596 (1996)).
Mutation analysis has shown that the phenylalanine

25   present at position 901 is required for core
stabilization of the Ras protein and that leucine
present at position 902 is important in the functional
interaction between GAP and Ras (Miao *et al.*, *J. Biol.*
Chem. 271:15322-15329 (1996); Hettich and Marshall,

30   *Cancer Res.* 54:5438-5444 (1994); Brownbridge et al., *J.*
Biol. Chem. 268:10914-10919 (1993)). The presence of
the FLP consensus motif in the Rin2 amino acid sequence

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provides additional support for the role of Rin2 as a
regulator of Ras.

As also described herein, the SY-A (Rin2) clone has
been used as a probe to screen a HT-29 human
5   adenocarcinoma cDNA library. Two overlapping cDNA
clones (HRIN2-1B1 (1.3 kb) and HRIN2-2B1 (2.9 kb)) have
been identified which are highly homologous to that of
the mouse Rin2 clone and which together represent the
full-length human Rin2 (Rabex-5) cDNA sequence. The
10   orientation of these overlapping clones is shown in
Figure 9. The nucleotide sequence of each clone is
shown in Figures 10A-B and 11A-C, respectively. The
determination of the complete sequence of these clones
can be carried out by the skilled artisan using methods
15   well known in the art.

The invention pertains to an isolated nucleotide
sequence encoding Rin2 protein. In one embodiment the
isolated nucleotide sequence is a mammalian nucleotide
sequence. In a particular embodiment the nucleotide
20   sequence is a murine or human nucleotide sequence. As
appropriate, nucleic acid molecules of the present
invention can be RNA, for example, mRNA, or DNA, such as
cDNA and genomic DNA. DNA molecules can be double-
stranded or single-stranded; single stranded RNA or DNA
25   can be either the coding (sense) strand or the non-
coding (antisense) strand. The nucleotide sequence can
encode a portion of the amino acid sequence of the Rin2
protein; alternatively, the nucleotide sequence can
include at least a portion of the Rin2 amino acid coding
30   sequence along with additional non-coding sequences,
such as introns and non-coding 3' and 5' sequences
(including regulatory sequences, for example).

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Additionally, the nucleotide sequence can be fused to a
marker sequence, for example, a sequence which encodes a
polypeptide to assist in isolation or purification of
the Rin2 protein. Such sequences include, but are not
5   limited to, those which encode a glutathione-S-
transfeRase (GST) fusion protein and those which encode
a hemaglutin A (HA) peptide marker from influenza.

As used herein, "isolated" is intended to mean that
the material in question exists in a physical milieu
10   distinct from that in which it occurs in nature and/or
has been completely or partially purified from other
transcribed sequences (as in a cDNA or RNA library).
Thus, an isolated gene or nucleotide sequence can
include a gene or nucleotide sequence which is
15   synthesized chemically or by recombinant means.
Recombinant DNA contained in a vector is included in the
definition of "isolated" as used herein. Also, isolated
nucleotide sequences include recombinant DNA molecules
in heterologous host cells, as well as partially or
20   substantially purified DNA molecules in solution. *In
vivo* and *in vitro* RNA transcripts of the DNA molecules
of the present invention are also encompassed by the
term "isolated" nucleotide sequences. Such isolated
nucleotide sequences are useful in the manufacture of
25   the encoded protein, as probes for isolating homologous
sequences (e.g., from other mammalian species), for gene
mapping (e.g., by *in situ* hybridization with
chromosomes), or for detecting expression of the *rin2*
gene in tissue (e.g., human tissue), such as by Northern
30   blot analysis.

The invention relates to nucleic acid molecules
comprising the nucleotide sequence of SEQ ID NO: 1 or

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the complement of SEQ ID NO: 1. The invention also
relates to nucleic acid molecules comprising the
nucleotide sequence of SEQ ID NO: 12 and/or SEQ ID NO:
13. That is, the nucleic acid molecule can comprise SEQ
5   ID NO: 12, SEQ ID NO: 13 or both SEQ ID NOS: 12 and 13.
The invention further relates to a nucleic acid molecule
comprising the nucleotide sequence of SEQ ID NO: 14
and/or SEQ ID NO: 15. That is, the nucleic acid
molecule can comprise SEQ ID NO: 14, SEQ ID NO: 15 or
10   both SEQ ID NOS: 14 and 15. In a particular embodiment,
the nucleic acid molecule comprises one or more
nucleotide sequences selected from the group consisting
of SEQ ID NOS: 12, 13, 14 and 15.

          The present invention also pertains to nucleotide
15   sequences which are not necessarily found in nature but
which encode Rin2 protein. Thus, DNA molecules which
comprise a sequence which is different from the
naturally-occurring nucleotide sequence but which, due
to the degeneracy of the genetic code, encode Rin2
20   protein are the subject of this invention. The
invention also encompasses variations of the nucleotide
sequences of the invention, such as those encoding
portions, analogues or derivatives of Rin2 protein.
Such variations can be naturally-occurring, such as in
25   the case of allelic variation, or non-naturally-
occurring, such as those induced by various mutagens and
mutagenic processes. Included variations include, but
are not limited to, addition, deletion and substitution
of one or more nucleotides which can result in
30   conservative or non-conservative amino acid changes,
including additions and deletions. Preferably, the
nucleotide or amino acid variations are silent or

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conserved, respectively; that is, they do not alter the
characteristics or activity of Rin2 protein.

    The invention described herein also relates to
fragments of the isolated nucleic acid molecules
5   described above. The term "fragment" is intended to
encompass a portion of a nucleotide sequence described
herein which is from at least about 25 contiguous
nucleotides to at least about 50 contiguous nucleotides
or longer in length; such fragments are useful as
10 probes, e.g., for diagnostic methods and also as
primers, and can encode functional portions of the Rin2
polypeptide. Particularly preferred primers and probes
selectively hybridize to the nucleic acid molecule
encoding Rin2 protein described herein. For example,
15 fragments which encode antigenic regions of the Rin2
protein described herein are useful.

    The invention also pertains to nucleotide sequences
which hybridize under medium stringency hybridization
conditions (e.g., for selective hybridization) to a
20 nucleotide sequence described herein. Appropriate
stringency conditions which are considered "medium
stringency" are known to those skilled in the art or can
be found in standard texts such as *Current Protocols in
Molecular Biology*, John Wiley & Sons, N.Y. (1989),
25 6.3.1-6.3.6. Preferred hybridization conditions allow
for specific hybridization of a nucleotide sequence to a
nucleotide sequence described herein. Such hybridizable
nucleotide sequences are useful as probes and primers
for diagnostic applications.
30   The invention pertains to nucleotide sequences
which have a substantial identity with the nucleotide
sequence of SEQ ID NO: 1; particularly preferred are

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nucleotide sequences which have at least about 75%,
preferably at least about 80%, more preferably at least
about 85%, and more preferably at least about 95%
identity with nucleotide sequences described herein.

5   Particularly preferred in this instance are nucleotide
sequences encoding polypeptides having at least one
activity of the novel Rin2 protein described herein, or
which hybridize with characteristic sequences of *rin2*
and can therefore be used as probes or primers. For

10   example, preferred nucleotide sequences encoding a
polypeptide having the same or similar biological
activity as the Rin2 protein, and nucleotide sequences
encoding a polypeptide with the same or similar
immunogenic or antigenic properties as the Rin2 protein

15   are within the scope of the invention. As used herein,
activities of the Rin2 protein include, but are not
limited to, catalytic activity, binding function,
antigenic function and oligomerization function.

This invention also pertains to an isolated protein

20   or polypeptide which is a novel Rin2 protein, such as
the protein or polypeptide encoded by any one or more of
SEQ ID NOS: 1, 12, 13 and 14. Rin2 protein
down-regulates the functional responses elicited by
FceRI aggregation in mast cells, including down-

25   regulating the amounts of preformed mediator (serotonin)
and cytokine (IL-6) released from these cells upon
FceRI-dependent stimulation. It may also down-regulate
responses elicited at other types of receptors (such as
TrkA receptors for NGF, c-kit receptors for SCF, etc.)

30   in response to receptor interaction with ligand or due
to auto-activation of the receptors (e.g., by activating
mutations).

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For example, an isolated protein of the invention
may be substantially isolated with respect to the
complex cellular milieu in which it naturally occurs.
In some instances, the isolated material will form part

5   of a composition (for example, a crude extract
containing other substances), buffer system or reagent
mix. In other circumstances, the material may be
purified to essential homogeneity, for example as
determined by PAGE or column chromatography such as

10 HPLC. According to the invention, the amino acid
sequence of the polypeptide can be that of the
naturally-occurring protein or can comprise alterations
therein. Such alterations include conservative or non-
conservative amino acid substitutions, additions and

15 deletions of one or more amino acids; however, such
alterations should preserve at least one activity of the
Rin2 protein, i.e., the altered or mutant protein should
be an active derivative of the naturally-occurring
protein. For example, the mutation(s) can preferably

20 preserve the three dimensional configuration of the
binding and/or catalytic site of the native protein.
The presence or absence of Rin2 activity can be
determined by various functional assays, as described
herein. Moreover, amino acids which are essential for

25 the function of the Rin2 protein can be identified by
methods known in the art. Particularly useful methods
include identification of conserved amino acids in the
family or subfamily of Ras-interference or Ras-
inhibiting proteins, site-directed mutagenesis and

30 alanine-scanning mutagenesis (for example, Cunningham
and Wells, *Science* 244:1081-1085 (1989)),
crystallization and nuclear magnetic resonance. The

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altered polypeptides produced by these methods can be
tested for particular biologic activities, including
immunogenicity and antigenicity.

Specifically, appropriate amino acid alterations
5   can be made on the basis of several criteria, including
hydrophobicity, basic or acidic character, charge,
polarity, size, the presence or absence of a functional
group (e.g., -SH or a glycosylation site), and aromatic
character. Assignment of various amino acids to similar
10 groups based on the properties above will be readily
apparent to the skilled artisan (see, for example,
Figure 4); further appropriate amino acid changes can
also be found in Bowie et al. (*Science 247*:1306-
1310(1990)).

15   The Rin2 polypeptide can also be a fusion protein
comprising all or a portion of the Rin2 amino acid
sequence fused to an additional component. Additional
components, such as radioisotopes and antigenic tags,
can be selected to assist in the isolation or
20 purification of the polypeptide or to extend the half
life of the polypeptide; for example, a hexahistidine
tag permits ready purification by nickel chromatography.

Also included in the invention are novel
polypeptides which are at least about 40% similar to the
25 Rin2 protein described herein. However, polypeptides
exhibiting lower levels of identity are also useful,
particularly if they exhibit high, e.g., at least about
40%, similarity over one or more particular domains of
the protein. For example, polypeptides sharing high
30 degrees of identity or similarity over domains necessary
for particular activities, including binding and
enzymatic activity, are included herein. As used

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herein, "similar" amino acids are intended to mean
identical amino acids or conserved substitutions of
amino acids.

        Polypeptides described herein can be isolated from
5   naturally-occurring sources, chemically synthesized or
recombinantly produced. Polypeptides or proteins of the
present invention can be used as a molecular weight
marker on SDS-PAGE gels or on molecular sieve gel
filtration columns using art-recognized methods.

10   The invention also provides expression vectors
containing a nucleic acid sequence encoding a
polypeptide which is a Rin2 polypeptide, operably linked
to at least one regulatory sequence. Many such vectors
are commercially available, and other suitable vectors
15 can be readily prepared by the skilled artisan.
"Operably linked" is intended to meant that the
nucleotide sequence is linked to a regulatory sequence
in a manner which allows expression of the nucleic acid
sequence in a host cell. Regulatory sequences are art-
20 recognized and are selected to produce a polypeptide
which is Rin2. Accordingly, the term "regulatory
sequence" includes promoters, enhancers, and other
expression control elements which are described in
Goeddel, *Gene Expression Technology: Methods in*
25 *Enzymology 185*, Academic Press, San Diego, CA (1990).
For example, the native regulatory sequences or
regulatory sequences native to a transformed host cell
can be employed. It should be understood that the
design of the expression vector may depend on such
30 factors as the choice of the host cell to be transformed
and/or the type of protein desired to be expressed. For
instance, the polypeptides of the present invention can

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be produced by ligating the cloned gene, or a portion
thereof, into a vector suitable for expression in either
prokaryotic cells, eukaryotic cells or both (see, for
example, Broach, et al., *Experimental Manipulation of*
5   *Gene Expression*, ed. M. Inouye (Academic Press, 1983) p.
83; *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed.
Sambrook *et al.* (Cold Spring Harbor Laboratory Press,
1989) Chapters 16 and 17). Typically, expression
constructs will contain one or more selectable markers,
10 including, but not limited to, the gene that encodes
dihydrofolate reductase and the genes that confer
resistance to geneticin, neomycin, tetracycline,
ampicillin, chloramphenicol, kanamycin and streptomycin
resistance.
15   Prokaryotic and eukaryotic host cells transfected
by the described vectors are also provided by this
invention. For instance, cells which can be transfected
with the vectors of the present invention include, but
are not limited to, bacterial cells such as *E. coli*
20 (e.g., *E. coli* K12 strains), *Streptomyces*, *Pseudomonas*,
Serratia marcescens and *Salmonella typhimurium*, insect
cells (baculovirus), including *Drosophila*, fungal cells,
such as yeast cells, plant cells and mammalian cells,
such as mast cells, PC12 cells, EL-4 T cells or other T
25 cells, Chinese hamster ovary cells (CHO) and COS cells.
     Thus, a nucleotide sequence derived from the
cloning of Rin2 described herein can be used to produce
a recombinant form of the protein via microbial or
eukaryotic cellular processes. Ligating the
30 polynucleotide sequence into a gene construct, such as
an expression vector, and transforming or transfecting
into hosts, either eukaryotic (yeast, avian, insect,

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plant or mammalian) or prokaryotic (bacterial cells),
are standard procedures used in producing other well
known proteins. Similar procedures, or modifications
thereof, can be employed to prepare recombinant proteins
5   according to the present invention by microbial means or
tissue-culture technology. Accordingly, the invention
pertains to the production of Rin2 proteins or
polypeptides by recombinant technology.

The proteins and polypeptides of the present
10   invention can be isolated or purified (e.g., to
homogeneity) from recombinant cell culture by a variety
of processes. These include, but are not limited to,
anion or cation exchange chromatography, ethanol
precipitation, affinity chromatography and high
15   performance liquid chromatography (HPLC). The
particular method used will depend upon the properties
of the polypeptide and the selection of the host cell;
appropriate methods will be readily apparent to those
skilled in the art.

20   The present invention also relates to antibodies
which bind a polypeptide which is Rin2. For instance,
polyclonal and monoclonal antibodies, including non-
human and human antibodies, humanized antibodies,
chimeric antibodies and antigen-binding fragments
25   thereof (Current Protocols in Immunology, John Wiley &
Sons, N.Y. (1994); EP Application 173,494 (Morrison);
International Patent Application WO86/01533 (Neuberger);
and U.S. Patent No. 5,225,539 (Winters)) which bind to
the described protein are within the scope of the
30   invention. A mammal, such as a mouse, rat, hamster or
rabbit, can be immunized with an immunogenic form of
Rin2 (e.g., Rin2 or a peptide comprising an antigenic

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fragment of Rin2 which is capable of eliciting an
antibody response). Techniques for conferring
immunogenicity on a protein or peptide include
conjugation to carriers or other techniques well known
5   in the art. The protein or polypeptide can be
administered in the presence of an adjuvant. The
progress of immunization can be monitored by detection
of antibody titers in plasma or serum. Standard ELISA
or other immunoassays can be used with the immunogen as
10 antigen to assess the levels of antibody.

Following immunization, anti-peptide antisera can
be obtained, and if desired, polyclonal antibodies can
be isolated from the serum. Monoclonal antibodies can
also be produced by standard techniques which are well
15 known in the art (Kohler and Milstein, *Nature 256*:495-
497 (1975); Kozbar et al., *Immunology Today 4*:72 (1983);
and Cole et al., *Monoclonal Antibodies and Cancer
Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). The
term "antibody" as used herein is intended to include
20 fragments thereof, such as Fab and $F(ab)_2$. Antibodies
described herein can be used to inhibit the activity of
Rin2 described herein, particularly *in vitro* and in cell
extracts, using methods known in the art. Additionally,
such antibodies, in conjunction with a label, such as a
25 radioactive label, can be used to assay for the presence
of the expressed protein in a cell from, e.g., a tissue
sample, and can be used in an immunoabsorption process,
such as an ELISA, to isolate the Rin2 protein. Tissue
samples which can be assayed include human tissues,
30 e.g., differentiated and non-differentiated cells.
Examples include, but are not limited to, heart, lung,
skin, skeletal muscle, bone marrow, thymus, lymph node,

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spleen, kidney, liver, brain, pancreas, gastrointestinal
tract, gonads (testes, ovaries), fibroblasts and
epithelium.

The present invention also pertains to
5   pharmaceutical compositions comprising polypeptides
described herein. For instance, a polypeptide or
protein, or prodrug thereof, of the present invention
can be formulated with a physiologically acceptable
medium to prepare a pharmaceutical composition. The
10   particular physiological medium may include, but is not
limited to, water, buffered saline, polyols (e.g.,
glycerol, propylene glycol, liquid polyethylene glycol)
and dextrose solutions. The optimum concentration of
the active ingredient(s) in the chosen medium can be
15   determined empirically, according to well known
procedures, and will depend on the ultimate
pharmaceutical formulation desired. Methods of
introduction of exogenous peptides at the site of
treatment include, but are not limited to, intradermal,
20   intramuscular, intraperitoneal, intravenous,
subcutaneous, oral, intranasal intrarectal,
intravaginal, and aerosol (for administration to the
respiratory tract). Other suitable methods of
introduction can also include gene therapy, rechargeable
25   or biodegradable devices and slow release polymeric
devices. The pharmaceutical compositions of this
invention can also be administered as part of a
combinatorial therapy with other agents.

While the intracellular events which lead to the
30   activation of cell proliferation, differentiation, or
functional responses (e.g., FceRI-dependent mast cell
mediator secretion) have been investigated in great

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detail, equally as interesting are the pathways which
can suppress such responses. For example, recent
evidence indicates that certain cell surface receptors,
such as FcγRIIb or gp149 in mast cells, can mediate
5   signals which can down-regulate the cell activation
signals which are transduced by other cell surface
receptors, including the mast cell FceRI (Scharenberg
and Kinet, *Cell* 87:961-964 (1996)). However, the work
described herein is directed to searching for
10 intracellular molecules which function to down-regulate
the signaling pathways in which Ras plays a role,
including those which govern the secretion of mast cell
mediators or the proliferative responses of neuronal
cells or other cell types. Ras plays a pivotal role in
15 signaling pathways which mediate diverse cellular
responses to a spectrum of receptor-ligand interactions
(Bourne *et al.*, *Nature* 348:125-132 (1990); Marshall,
FASEB J. 9:1311-1318 (1995)). Recent studies have shown
that the functional activation of mast cells which is
20 induced by the aggregation of FceRI by IgE and specific
antigen is associated with the activation of Ras and its
effector pathways (Beaven and Baumgartner, *Curr. Opin.
Immunol.* 8:766-772 (1996); Jabril-Cuenod *et al.*, *J.
Biol. Chem.* 271:16268-16272 (1996)).
25   FceRI-mediated Ras activation in mast cells is
accompanied by activation of the Shc-Grb2-Sos pathway,
which precedes Ras activation, as well as the Ras
effector pathway of Raf-1/Erk-MAP kinases (Jabril-Cuenod
et al., *J. Biol. Chem.* 271:16268-16272 (1996)). Recent
30 studies with rat RBL-2H3 mast cells have demonstrated
that FceRI-induced Ras activation results in a
transcription activation mediated by Elk-1 and NFAT

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(Turner and Cantrell, *J. Exp. Med.* *185*:43-53 (1997)).
The Elk-1 transcription factor exerts its effects by
interacting with the serum response element, which is a
regulatory component of early response genes such as

5   c-*fos* (Treisman, *Curr. Opin. Genet. Dev.* 4:96-101
(1994)), whereas members of the NFAT transcription
factor family form regulatory complexes with inducible
nuclear factor such as Fos or Jun (Rao, *Immunol. Today*
15:274-281 (1994)). Such complexes interact with

10 cytokine gene promoters and activate transcriptions of
genes for IL-2, IL-4, GM-CSF, and TNF-α (Rao, *Immunol.
Today* *15*:274-281 (1994)). In mast cells, NFAT is
implicated in the regulation of transcription of the
IL-4 gene (Weiss *et al.*, *Mol. Cell. Biol.* *16*:228-235

15 (1996)). Interestingly, the activation of the
Raf-1/MEK/Erk cascade is necessary and sufficient for
the activation of Elk-1 in mast cells, whereas the
induction of NFAT by FcϵRI stimulation requires the
activity of Rac-1 (Turner and Cantrell, *J. Exp. Med.*

20 *185*:43-53 (1997)). Earlier studies have shown that Rin1
interacts directly with Ras in a manner that is
characteristic of Raf-1, suggesting that Rin1 is a
negative downstream effector of Ras (Han and Colicelli,
Mol. Cell. Biol. *15*:1318-1323 (1995)).

25   As shown herein, reduced expression of Rin2 can
dramatically potentiate the downstream signaling
responses mediated by Ras. In mouse mast cells
transfected with the *rin2* antisense expression vector,
the induction of early-response gene expression and the

30 activation of MAP kinase activity in response to FcϵRI
crosslinking are significantly augmented, as compared
with those observed in mast cells transfected with a

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control vector. These results support a role of Rin2 as
a negative effector/regulator of Ras. Furthermore,
reduced expression of Rin2 can markedly enhance the
functional responses elicited by FcεRI aggregation in
5   mouse mast cells; the expression of antisense *rin2* in
mouse mast cells can significantly increase the amounts
of preformed mediators (e.g., 5-HT) and cytokine (e.g.,
IL-6) released from these cells upon FcεRI-dependent
stimulation. These data indicate that Rin2 can
10   negatively regulate the Ras-mediated signaling responses
in mast cells that lead to both the release of preformed
mediators and the transcription of cytokine genes and
the secretion of the corresponding products.
Stimulation of mast cells by IgE and antigen results in
15   Ras activation followed by a rapid and transient
induction of *rin2* mRNA expression. Rin2 appears to
exert its effects by down-regulating the signaling and
functional responses elicited by FcεRI aggregation.
Thus, during the period subsequent to mast cell
20   activation, Rin2 may function as a cellular switch in
turning off ongoing signaling initiated by Ras
activation. In mast cells, such a regulatory process
may be important as a feedback mechanism to restore the
perturbed cellular environment to its normal basal
25   physiological state after the cessation of cell
activation.

     Furthermore, *rin2* mRNA expression is significantly
elevated in mouse mast cells stimulated with stem cell
factor (SCF), with a kinetics of activation that is very
30   similar to that which is observed in mast cells after
FcεRI-dependent stimulation. SCF, the cognate ligand
for the c-kit tyrosine kinase receptor, is a major mast

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cell growth factor which has been shown to be critical
in the maturation and development/function of rodent and
human mast cells (Galli *et al.*, *Adv. Immunol.* 55:1-96
(1994)). The interaction of SCF with the c-kit tyrosine
5   kinase receptor has been shown to induce an activation
of Ras and its effector pathways via the activation of
the Shc-Grb2-Sos signaling pathway (Duronio *et al.*,
Proc. Natl. Acad. Sci. USA 89:1587-1591 (1992); Tauchi
et al., *J. Exp. Med.* 179:167-175 (1994)).

10   As shown herein, nerve growth factor (NGF) can also
elicit a rapid and sustained induction of expression of
rin2 mRNA transcript through it receptor. NGF has been
shown to induce neuronal differentiation and cessation
of cell proliferation in PC12 cells via its interaction
15   with TrkA receptor tyrosine kinase (Barbacid, *J.
Neurobiol.* 25:1386-1403 (1994); Bothwell, *Ann. Rev.
Neurosci.* 18:223-253 (1995)). PC12 cells are a commonly
used *in vitro* model of neuronal development and function
(Greene and Tischler, *Proc. Natl. Acad. Sci. USA*
20   73:2424-2428 (1976)). Previous studies have indicated
that NGF stimulation of TrkA receptors in PC12 cells
leads to Ras activation and the activation of the
Raf-1/MEK/MAP kinases effector pathway (Thomas *et al.*,
Cell 68:1031-1040 (1992); Wood *et al.*, *Cell* 68:1041-50
25   (1992)). Results presented herein show that PC12 cells
which have been transfected with the pBK-CMV-SYA-AS
vector and therefore have reduced expression of Rin2 can
still differentiate in response to NGF stimulation.
However, these transfected cells exhibit a higher rate
30   of cell proliferation than that detected in control PC12
cells. This effect is observed in both unstimulated
transfected PC12 cells and transfected PC12 cells

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stimulated with NGF. NGF is also known to have a transient proliferative effect on PC12 cells, which precedes the better known anti-mitogenic effects of NGF on these cells. Thus, by suppressing Ras-dependent

5   proliferative effects, Rin2 may play a role in mediating the anti-mitogenic effects of NGF on these neuronal cells. Taken together, these data indicate that the expression of the *rin2* gene transcript can be differentially regulated in mast cells and in PC12 cells

10 via different ligand-receptor interactions.

As disclosed herein, activation of a mouse T cell lymphoma line (EL-4 cells) via the T cell receptor, by stimulating the cells with antibodies directed against CD3 and CD28, also elicits a rapid and transient

15 induction of expression of *rin2* mRNA transcript. T cells are essential for the expression of an adequate immune response, but inappropriate activation of T cells is thought to contribute to a wide variety of diseases with "autoimmune" components, such as autoimmune (Type

20 I) diabetes mellitus, rheumatoid arthritis, ankylosing spondylitis, sarcoidosis, Sjögren's syndrome, multiple sclerosis, inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis), dermatomyositis, scleroderma, polymyositis, systemic lupus erythematosus,

25 biliary cirrhosis, autoimmune thyroiditis, and autoimmune hepatitis, as well as many dermatological disorders, including psoriasis, contact sensitivity and atopic dermatitis (Schwartz, Autoimmunity and Autoimmune Diseases, in *Fundamental Immunology*, Third Edition,

30 Paul, ed. (Raven Press, NY, pp. 1033-1097 (1993)); Rich, *Clinical Immunology*: Principles and Practice, Mosby, St. Louis (1996)). T cells are also important in the

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process of tissue graft rejection, such as grafts of
bone marrow, hematopoietic stem cells, skin, heart,
lung, liver, kidney, pancreas and intestine, among
others, and in graft-versus-host disease; T cells can
5   also undergo neoplastic transformation, resulting in the
development of leukemias or lymphomas. The findings
with EL-4 T cells disclosed herein support the role of
Rin2 as a negative Ras effector/regulator mediating
diverse cellular responses in T cells. Taken together
10 with the results disclosed herein regarding the role of
Rin2 in regulating cellular responses in mast cells and
PC12 adrenal pheochromocytoma cells, the findings in EL-
4 T cells are also consistent with the hypothesis that
Rin2 is a negative Ras effector/regulator mediating
15 diverse functional responses in different cell types.
There are likely to be many other cell types in which
Rin2 may down-regulate Ras-dependent functional
responses, since Ras is involved in influencing
functional responses in diverse cell types (Marshall,
20 *FASEB J 9*:1311-1318 (1995)). Additionally, cells which
express the FceRI receptor, T cell receptor, TrkA and c-
kit, as well as diverse other receptors which signal by
mechanisms which are influenced by Ras, can have their
Ras-dependent functional responses negatively regulated
25 by Rin2.

It will be apparent to the skilled artisan that the
identification of the novel protein Rin2, which can
inhibit Ras-dependent cellular signaling, has many
commercial applications. The Ras effector signaling
30 pathways have been shown to be important in mediating
signal transduction from the cell membrane to the

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nucleus in many oncogenic, mitogenic, and developmental
processes.

As a result of Rin2's ability to inhibit IgE and
antigen-dependent release of mediators from mast cells,
5   compositions which can enhance Rin2 expression and/or
function (e.g., Rin2 itself or other compounds), or
which mimic these actions (e.g., molecules synthesized
based on the structure/functional characteristics of the
Rin2 protein), can be used to inhibit mediator release
10 from effector cells in disorders such as asthma and
other allergic diseases (e.g., hay fever and atopic
eczema), thus ameliorating these disorders.

The ability of Rin2 to negatively regulate
Ras-dependent signal transduction in other cell types
15 (in addition to mast cells) suggests that compounds
(e.g., isolated Rin2, Rin2 "mimics" or agents that can
enhance expression of native Rin2) that are able to
block such Ras-dependent cellular signaling pathways can
produce clinically desirable effects. Mutated forms of
20 cellular *Ras* genes, resulting in oncogenic activation of
Ras proteins, have been found to be among the most
common genetic abnormalities associated with human
cancer. The early stage in multistage tumor progression
has been associated with Ras activation, and disruption
25 of the oncogenic *Ras* genes in colon cancer cell lines
has been shown to revert the cells to a more regulated
state of growth (Shirasawa et al., *Science* 260:85-88).
Thus, the *rin2* cDNA clone can be used to identify
compounds that can block Ras-induced cell transformation
30 *in vitro*. These compounds can include isolated Rin2
(e.g., overexpression of Rin2), derivatives (modified
forms) of Rin2, and compounds identified by another

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method, e.g., computer modeling of compounds designed
with reference to structures and/or characteristics of
Rin2 and synthesized by known methods (e.g., chemical
synthesis, peptide chemistry). These compounds can be
5   tested for their ability to function as Ras-specific
anti-neoplastic agents for the treatment of cancer, or
as Ras-specific agents which inhibit other examples of
clinically unwanted, but non-neoplastic, Ras-dependent
cellular proliferation.

10   Mutations in the genes encoding other molecules
which are involved in Ras-dependent intracellular
signaling have been linked to human diseases with a
genetic component, such as some cases of melanoma. The
rin2 cDNA clone can be used to map, identify and isolate
15   or clone the human counterpart of the *rin2* gene and,
ultimately, to assess whether mutations of Rin2 and/or
genes identified using Rin2 are linked to known human
diseases.

The *rin2* cDNA clone can be used in a yeast
20   two-hybrid system to identify other novel cellular
proteins that interact with Rin2 and/or Ras and thus
regulate the signaling responses along Ras effector
pathways. Using a yeast two-hybrid system (Fields and
Sternglanz, *Trends Genet. 10*:286-292 (1994)), specific
25   regions of the Rin2 protein that interact with Ras can
be identified. The elucidation of the mechanism of
protein-protein interaction between Rin2 and Ras will be
useful in the development of small molecule drugs that
interact specifically with Ras and inhibit or enhance
30   its function.

Using the *rin2* cDNA clone, the Rin2 protein can be
produced using a protein expression system. The

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partially purified Rin2 protein, or a synthetic peptide
based on the known Rin2 amino acid sequence, can be used
to raise Rin2-specific antibodies which can be utilized
as laboratory reagents for studying the Ras effector

5   pathways, as well as "humanized" antibodies for
potential clinical applications. Rin2 is an
intracellular protein. Thus, small fragments of
antibodies against Rin2, perhaps specifically modified
to enhance their ability to identify specific cell types

10   and to pass through the cells' surface (plasma
membrane), may be required to gain appropriate access to
the intracellular Rin2 target.

However, other natural or newly-synthesized
chemicals that, because of their properties, can readily

15   enter cells and achieve intracellular concentrations
which are sufficient to permit the expansion of the
desired interactions with Rin2, can be identified via
their ability to interact with Rin2, Rin2 fragments or
Rin2-mimicking compounds, in various in vitro systems.

20   Rin2-mimicking compounds are defined herein as peptide
or protein compounds which may be unrelated to Rin2 in
amino acid sequence, or non-peptide based compounds,
which express sufficient structural similarity to native
Rin2 or fragments thereof to permit such compounds to be

25   used for initial screening of compounds, including those
generated through recombinatorial chemistry approaches,
to identify those which can interact with active Rin2
sufficiently to enhance or inhibit its function.

As used herein, "inhibition" is intended to

30   encompass any reduction in the quality or quantity of
the assessed activity, including complete abolishment
thereof. The term "enhancement" as used herein is

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intended to mean any increase in the quality or quantity
of the assessed activity, including increases resulting
from mimicking of an assessed activity. As used herein,
"alteration"" is intended to mean either inhibition or
5   enhancement of the quality or quantity of the assessed
activity. As used herein, "mimicking" is intended to
mean that the assessed activity is substantially similar
to the corresponding activity of Rin2.

          Depending on the circumstances, it may be desirable
10   either to inhibit or to enhance Rin2 activity, in order
to enhance or to suppress, respectively important Ras-
dependent signaling pathways. Several examples of the
inhibition of Rin2 activity, resulting in reduced Rin2-
dependent inhibition of Ras-dependent pathways, with
15   subsequent enhancement of such pathways, can be
envisioned. Enhancement of Ras-dependent pathways
promoting the proliferation and functional activation of
cells involved in wound healing, including the
development of new blood vessels (i.e., angiogenesis) in
20   areas of ischemic tissue (e.g., in tissues such as
cardiac or skeletal muscle, which are ischemic because
of the consequences of arteriosclerosis), can have great
therapeutic benefit. Enhancement of the Ras-dependent
pathways which contribute to the proliferation of, and
25   production of mediators and cytokines by, T cells and
mast cells, and other cell types activated through the
same or distinct receptors, may be of great benefit in
diseases that are associated with diminished numbers
and/or function of these cells, such as in patients
30   infected with the human immunodeficiency virus (HIV), or
receiving treatment with radiation or chemical agents
for the treatment of cancer. Enhancement of Ras-

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dependent signaling in neuronal cells in response to NGF
or other neurotrophic factors may be desirable in
settings characterized by neuronal loss, or when neural
regeneration is an objective (Satoh et al., Mol. Cell.
5   Biol. 7:4553-4556 (1987); Szeber'nyi et al., Mol. Cell.
Biol. 10:5324-5332 (1990); Qiu and Green, Neuron 7:937-
946 (1991); Muroya et al., Oncogene 7:277-281 (1992)).
For example, Ras-dependent pathways, such as the
functional responses elicited by FceRI aggregation, can
10 be inhibited by enhancing the activity of Rin2. Agents
which enhance Rin2 activity include, but are not limited
to, isolated Rin2, Rin2 mimics or agents that can
enhance expression of native Rin2.

    In contrast, enhancement of Rin2 activity,
15 resulting in increased Rin2-dependent inhibition of Ras-
dependent pathways, with subsequent inhibition of such
pathways, can also be useful. For example, it may be
desirable to inhibit TCR- and Ras-dependent pathways
promoting the proliferation and functional activation of
20 cells involved in tissue graft rejection, graft-versus-
host disease, and autoimmune disorders or T cell-
associated disorders, e.g., autoimmune (Type I) diabetes
mellitus, rheumatoid arthritis, ankylosing spondylitis,
sarcoidosis, Sjögren's syndrome, multiple sclerosis,
25 inflammatory bowel disease (i.e., Crohn's disease and
ulcerative colitis), dermatomyositis, scleroderma,
polymyositis, systemic lupus erythematosus, biliary
cirrhosis, autoimmune thyroiditis, and autoimmune
hepatitis, as well as many dermatological disorders,
30 including psoriasis, contact sensitivity and atopic
dermatitis. In asthma and allergic diseases such as hay
fever and atopic dermatitis, it is desirable to inhibit

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the Ras-dependent functional responses induced by FceRI
aggregation in cells bearing the FceRI receptor, e.g.,
mast cells. Specifically, the intracellular signaling
(e.g., activation of MAP kinase) and cellular secretory

5   responses (e.g., release of preformed mediators, such as
5-HT, and cytokines, such as IL-6) can be inhibited by
enhancing Rin2 activity. Additionally, enhancement of
Rin2 activity can also be used to inhibit cellular
proliferation through the TrkA receptor, e.g., in PC12

10 cells, and to inhibit Ras-dependent cellular
proliferation, including cancers or other unwanted
neoplastic or non-neoplastic proliferation of cells.

    Alteration of Rin2 activity can be accomplished by
increasing or decreasing the amount of Rin2 protein

15 present to inhibit or enhance, respectively, Ras-
dependent pathways, the activity of the Rin2 protein, or
both. For example, the activity of the Rin2 protein
described herein can be enhanced by overexpressing
isolated Rin2 protein from an appropriate expression

20 construct in a host cell. Alternatively, a cell
population of interest can be treated with one or more
agents, such as SCF, that enhance expression or activity
of native Rin2 present in the cell population.
Additionally, compounds which mimic Rin2 activity are

25 useful in applications in which native or isolated Rin2
are useful. Useful compounds will be able to mimic at
least one appropriate activity of Rin2, e.g., binding
activity, catalytic activity, and include fragments and
derivatives of Rin2. Alternatively, Ras-dependent

30 pathways can be enhanced by inhibiting the activity of
Rin2. Agents which inhibit Rin2 activity include, but

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are not limited to, agents that can inhibit expression
of native Rin2 and agents which bind native Rin2.

The invention also pertains to methods of
inhibiting functional responses induced by Ras-dependent

5   signaling pathways in a mammal by administering to the
mammal an effective amount of an agent which enhances
the activity of Rin2. The invention also pertains to
methods of inhibiting cellular proliferation in a mammal
by administering to the mammal an effective amount of an

10   agent which enhances the activity of Rin2. The
invention also encompasses methods of inhibiting Ras-
dependent cellular proliferation, including cancers,
neoplastic and non-neoplastic proliferation, in a mammal
by administering to the mammal an effective amount of an

15   agent which enhances the activity of Rin2.

The invention also pertains to methods of enhancing
functional responses induced by Ras-dependent signaling
pathways in a mammal by administering to the mammal an
effective amount of an agent which inhibits the activity

20   of Rin2.

The present invention also relates to an assay for
identifying agents which alter the activity of Rin2,
such as agents which enhance or inhibit the activity of
Rin2. For example, a cell containing the Rin2 protein,

25   or an active fragment or derivative thereof, can be
activated via a specific receptor that induces signaling
that is modulated by Rin2, in the presence of an agent
to be tested, and the level of Rin2 activity can be
assessed; in a preferred embodiment, the level of Rin2

30   activity can be assessed and compared with the
corresponding level in a control in the absence of the
agent to be tested. The activity of Rin2 protein can be

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assessed directly, e.g., by assessing the expression
levels of the protein, or indirectly, such as by
assessing functional responses e.g., activation of MAP
kinase activity or secretion of 5-HT or IL-6. The cell
5   containing Rin2, or a derivative or portion thereof
having Rin2 activity, can be contacted directly or
indirectly with the stimulus in the presence of the
agent to be tested. Enhancement of Rin2 activity, or an
increase in the level of Rin2 activity relative to a
10   control, indicates that the agent is an agonist of Rin2
activity. Similarly, inhibition of Rin2 activity, or a
decrease in the level of Rin2 activity relative to a
control, indicates that the agent is an antagonist of
Rin2 activity.

15   The present invention also relates to novel agents
identified by the assay described above. Agents
identified by the assay described herein may enhance
(e.g., prolong or increase) or inhibit (e.g., shorten or
decrease) the activity of the Rin2 protein.

20   The following Examples are offered for the purpose
of illustrating the present invention and are not to be
construed to limit the scope of this invention. The
teachings of all references cited herein are hereby
incorporated herein by reference.

25

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EXAMPLES

MATERIALS AND METHODS

Cytokines, cDNA Probes and Antibodies

Recombinant mouse IL-3 was purchased from BioSource

5   International (Camarillo, CA). The c-*fos* probe is a 1.2

kb HindIII/EcoRI mouse cDNA fragment generously provided

by Dr. Brent Cochran. The cDNA probes were labeled with

$[\alpha-^{32}P]$ dCTP (6000 mCi/mmol) (New England Nuclear,

Boston, MA) using the exo(-) Klenow fragment of DNA

10   polymeRase I with random nonamer primers (Stratagene, La

Jolla, CA). Purified mouse 2.5 S NGF was purchased from

Upstate Biotechnology (Lake Placid, NY). For T-cell

activation experiments, purified hamster anti-mouse CD3e

and anti-mouse CD28 monoclonal antibodies were purchased

15   from Pharmingen (San Diego, CA).

Cell Culture

Bone marrow-derived cultured mast cells (BMCMCs)

were obtained by maintaining the femoral bone marrow

20   cells of 4-6 week-old BALB/c mice in suspension in

IL-3-containing conditioned medium, consisting of 10%

heat-inactivated fetal calf serum (FCS) (Sigma Chemical

Company, St. Louis, MO), 5×10^{-5} M 2-mercaptoethanol

(Sigma) and 2 mM L-glutamine (GIBCO Laboratories, Grand

25   Island, NY) in Dulbecco's Modified Eagle's Medium (DMEM;

GIBCO Laboratories, Grand Island, NY) (complete medium)

supplemented with 20% (v/v) of either supernatants from

Concanavalin A-activated spleen cells or WEHI-3

cell-conditioned medium (Nabel et al., *Nature*

30   *291*:332-334 (1981); Galli et al., *J. Cell Biol.*

95:435-444 (1982)). The cells were resuspended in fresh

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conditioned medium 1-2 times a week. After 4-5 weeks,
at least 95% of cells that remained in the cultures were
identifiable as mast cells, as determined by neutral red
or May-Grünwald/Giemsa staining. C1.MC/C57.1 cells, a
5   cloned growth factor-independent mouse mast cell line of
BALB/c origin (Young et al., *Proc. Natl. Acad. Sci. USA*
84:9175-9179 (1987); Tsai et al., *FASEB J. 10*, Abstract
A1268 (1996)), were maintained in complete medium
lacking exogenous growth factors. PC12 cells, a rat
10 adrenal pheochromocytoma cell line, were obtained from
American Type Culture Collection (ATCC No. CRL1721).
They were grown in RPMI 1640 medium supplemented with
10% horse serum (HS) and 5% FCS. EL-4 cells, a mouse
lymphoma T cell line, were obtained from ATCC (ATCC No.
15 TIB39) and grown in DMEM medium supplemented with 10%
HS.

FcεRI Cross-Linking

       The IgE anti-DNP mAb-producing hybridoma
20 H1-DNP-ε-26 (Liu et al., *J. Immunol. 124*:2728-2737
(1980)), generously provided by Drs. F.-T. Liu and D.H.
Katz, was used to generate ascites. BMCMC or
C1.MC/C57.1 mast cells were sensitized with the ascites
diluted 500-fold in complete medium (final IgE
25 concentration of approximately 8 μg/ml) for 2 hours at
37°C, washed with medium, then resuspended with medium
containing DNP_{30-40}-HSA (Sigma) at 50 ng/ml at 37°C.
These conditions of sensitization with IgE and
stimulation with specific antigen have been shown to be
30 adequate for the activation of such cells for [^{3}H] 5-HT

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release (Gordon and Galli, *J. Exp. Med.* 174:103-107
(1991)).

mRNA Differential Display

5   Differential display was performed using the RNAmap
KitA (GenHunter, Nashville, TN) according to the
manufacturer's specifications. Specifically, total RNA
was extracted from cultured cells using the RNAzol B
method (Biotecx Laboratories, Houston, TX) and

10   subsequently digested with DNaseI (1 unit/10 μg)
(Promega, Madison, WI) for 30 minutes at 37°C.
Approximately 2 μg of digested RNA was subjected to the
reverse transcription reaction with one of the four $T_{12}MN$
anchored primers (N=G, A, T, or C) at 65°C for 5

15   minutes, at 37°C for 60 minutes, and then at 95°C for 5
minutes. The MMLV reverse transcriptase (100 units) was
added after 10 minutes of incubation at 37°C. The
resulting cDNAs were further amplified by PCR (final
volume, 20 μl) with GeneAmp 1XPCR Buffer (Perkin Elmer,

20   Foster City, CA), 2 μMdNTPs, 0.2 μM AP-primers (AP-1:
5'-AGCCAGCGAA-3' (SEQ ID NO: 3); AP-2: 5'-GACCGCTTGT-3'
(SEQ ID NO: 4); AP-3: 5'-AGGTGACCGT-3' (SEQ ID NO: 5);
AP-4: 5'-GGTACTCCAC-3' (SEQ ID NO: 6); and AP-5:
5'-GTTGCGATCC-3' (SEQ ID NO: 7)), 1 μM $T_{12}MN$ primer (the

25   same primer used in the respective reverse transcription
reaction), 2 μl reverse transcription mix, 1 μl ^{35}S-dATP
(1200 Ci/mmol) (NEN, Boston, MA), and 1 unit AmpliTaq
DNA polymeRase (Perkin Elmer) at 94°C for 30 seconds,
40°C for 2 minutes, and 72°C for 30 seconds for 40

30   cycles, followed by an additional 5 minutes at 72°C.
3.5 μl of each sample was run on 6% sequencing gels.

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Differentially-expressed PCR products were excised from
the gel, extracted with water, and reamplified by PCR
using the same set of primers. The reamplified PCR
segments were ^{32}P-labeled as probes for Northern blot
5   analysis, or subcloned into pGEM-T Vector (Promega) and
sequenced using Sequenase version 2.0 (U.S. Biochemical,
Cleveland, OH).

Northern Hybridization

10   Total RNA was prepared from cultured cells or from
mouse tissues using the RNAzol B method (Biotecx)
according to the manufacturer's specifications; 10 or 15
μg of total RNA were loaded in each lane and
electrophoresed in 1% agarose-formaldehyde denaturing
15   gel, and then transferred onto Zetabind nylon membrane
(Cuno, Meriden, CT). RNA blots were hybridized at 42°C
for 16-18 hours with 10^6 cpm/ml of ^{32}P-labeled cDNA
probes, washed at 42°C to a final stringency of 0.2X
SSC, then exposed to Kodak XAR-5 films at -80°C.
20

cDNA Library Screening

The 60-4 probe isolated from the differential
display studies (see Results) was radiolabeled and used
as a probe to screen a BMCMC cDNA library, which had
25   been constructed in our laboratory using the Uni-ZAP XR
Vector (Stratagene, La Jolla, CA). One positive clone,
SY-6, with 1.1 kb insert was isolated. To further
isolate the full-length cDNA, the SY-6 clone was used as
a probe to screen a mouse brain cDNA library in Uni-ZAP
30   XR Vector purchased from Stratagene. Both libraries
were screened by filter replica hybridization, and

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plaque-purified phages were isolated with three rounds
of screening. *In vivo* excision of plasmids from the
lamda vector was performed using ExAssist helper phage
and SOLR recipient cells (Strategene).

5

In Vitro Transcription/Translation

Approximately 1 μg of the pBluescript SK (-)
containing the full-length SY-A cDNA clone was
transcribed and translated *in vitro* using the TNT T_3

10   Coupled Reticulocyte Lysate System (Promega). Proteins
were labeled in the translation step with 40 μCi of [^{35}S]
methionine (1,000 Ci/mmol) (Amersham, Arlington, IL),
run on an 8% SDS/PAGE, and visualized by
autoradiography.

15

Rin2 Antibody and Western Blotting

Anti-Rin2 antibodies were prepared by immunizing
rabbits with a synthetic peptide corresponding to the 16
N-terminal residues of murine Rin2 (KSERRGIHVDQSELLC;

20   SEQ ID NO: 16) conjugated to keyhole limpet hemacyanin.
The antibody was affinity-purified on a peptide column
using a cysteine residue of the peptide coupled to an
iodoacetamide on Sepharose beads. Cells were lysed in
lysis buffer (20 mM Tris 7.5, 150 mM NaCl, 1 mM EDTA, 1

25   mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1
mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1
mM PMSF). Resulting cell lysates were subjected to 12%
SDS-PAGE and electroblotted. Membranes were incubated
with the anti-Rin2 antibody (1:500), and the antigen-

30   antibody complexes were visualized with anti-rabbit IgG

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secondary antibody using enhanced chemiluminescence
(ECL).

Yeast Two-Hybrid Assay

5   The protein-protein interactions between murine
Rin2 and the murine or yeast Ras protein were assessed
using the MATCHMAKER Two-Hybrid System1 (Clontech)
according to the instructions supplied by the
manufacturer. The entire open reading frame, or

10   segments of the open reading frame of the SY-A clone was
subcloned, in frame, into the pACT2 vector. The murine
H-Ras cDNA was generated from total RNA of C1.MC/C57.1
by RT-PCR using the 5' primer
GCGGAATTCATGACAGAATACAAGCTTGTG (SEQ ID NO: 17) and the

15   3' primer GACGGATCCCTCAGGACAGCACACACTTGC (SEQ ID NO: 18)
and subcloned into pAS2-1. The yeast Ras2p cDNA was
generated from *S. cerevisiae* poly A+ RNA (Clontech) by
RT-PCR using the 5' primer CGATGTCGACCATGCCTTTGAACAAGTCG
(SEQ ID NO: 19) and the 3' primer

20   GATAGGATCCACCCGATCCGCTCTTG (SEQ ID NO: 20) and subcloned
into pAS2-1. A Cys-to-Ser mutation at residues 318 of
Ras2p, which suppresses palmitolylation, was constructed
using the QuikChange Site-Directed Mutagenesis Kit
(Stratagene). Y187 yeast strain was cotransformed with

25   the constructs described above and the transformants
were selected on -Leu/-Trp plates. β-galactosidase
activity was determined for colonies that appeared after
3-4 days by a qualitative colony-lift filter assay using
X-gal as substrate and a quantitative liquid culture

30   assay using CPRG as substrate.

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Generation of Stable Transfectants

The SY-A cDNA clone was digested with XbaI and XhoI restriction enzymes, and the entire full-length cDNA insert released was ligated to the pBK-CMV expression

5   vector (Stratagene) to generate the *rin2* antisense expression plasmid, pBK-CMV-SYAS. Plasmid pBK-CMV-SYAS and the vector control plasmid pBK-CMV were transfected into C1.MC/C57.1 mast cells or PC12 cells using LIPOFECTAMINE Reagent (Life Technologies, Gaithersburg,

10 MD) according to the manufacturer's instructions. For C1.MC/C57.1 cells, transfected cells were selected in Geneticin (G418) (Life Technologies) at 2 mg/ml for 10 days and then at 0.8 mg/ml thereafter. G418-resistant mast cell colonies were observed approximately two weeks

15 after transfection. For PC12 cells, transfected cells were selected in 0.6 mg/ml G418 and G418-resistant colonies were observed six weeks after transfection.

Measurement of Mediator Release

20   The release of 5-HT from mast cells was measured as previously described (Coleman et al., J. Immunol. 150:556-562 (1993)). Specifically, ^{3}H-hydroxytryptamine creatinine sulfate (25.2 Ci/ mmol) (NEN) was added to mast cells 2 hours before stimulation. The cells were

25 washed to remove unincorporated ^{3}H-5-HT and stimulated with IgE and specific antigen as described above. Release of radioactivity in supernatant fractions (150 μl) was determined after stimulation, and net percentage release was calculated as [(a-b)÷c] X 100, where "a" is

30 radioactivity released by stimulated cells, "b" is radioactivity released by unstimulated cells, and "c" is

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total incorporated cellular radioactivity that was
measured after cells were lysed in 0.05% Triton X-100
(Sigma). IL-6 release was assayed using enzyme-linked
immunosorbent assay ELISA kits (ENDOGEN, Boston, MA)
5   according to the manufacturer's instructions. Release
of β-hexosaminidase from transfectants was measured on
an ELISA reader using p-nitrophenyl-N-acetyl-β-D-
glucosamine (Sigma) as the substrate as described by
Berger in Immunology Methods Manual Vol. 3 (ed.
10   Lefkovits, I.):1436-1440 (Academic Press, San Diego,
1997).

Kinase Assays

The p44/p42 Erk-MAP kinase activity was assayed
15   using the MAP Kinase Assay Kit (New England Biolabs,
Beverly, MA). Specifically, cells were lysed by
sonication in 0.5 ml of cell lysis buffer (20 mM Tris
7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100,
2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1
20   mM Na_3VO_4 1 μg/ml leupeptin, 1 mM PMSF). 0.2 ml of cell
lysate was then incubated with the phospho-specific
p44/p42MAP kinase antibody (1:100 dilution) overnight at
4°C and subsequently with Protein A/G PLUS-Agarose
(Santa Cruz Biotechnology, Sant Cruz, CA) for 3 hours at
25   4°C. Immunoprecipitates were incubated in 50 μl 1X
kinase buffer (25 mM Tris 7.5, 5 mM β-glycerolphosphate,
2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) supplemented with
100 mM ATP and 1 μg of Elk1 fusion protein substrate for
30 minutes at 30°C. The reaction mixture was subjected
30   to 12% SDS-PAGE gel electrophoresis, and then
electroblotted onto Immobilon-P transfer membrane

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(Millipore, Bedford, MA). Membranes were incubated with
the phospho-specific Elk1 (Ser383) antibody (1:1000
dilution), and the antibody was visualized with
horseradish peroxidase-conjugated anti-rabbit IgG
5 secondary antibody using the Phototope-HRP Western
Detection Kit (New England Biolabs, Beverly, MA). The
films were subsequently scanned using a densitometer
(Molecular Dynamics, Sunnyvale, CA) and specific signals
for phospho-Elk1 were quantitated by ImageQuant software
10 (Molecular Dynamics).

The activation of JNK activity was assayed using
the SAPK/JNK Assay Kit (New England BioLabs).
Specifically, total JNK was immunoprecipitated using an
N-terminal c-Jun (1-89) fusion protein bound to
15 glutathione sepharose beads and incubated with ATP in
the presence of kinase buffer. The phosphorylation of
c-Jun by JNK was measured by Western blotting using a
phospho-specific c-Jun antibody.

The activation of p38 MAP kinase activity was
20 assayed using the p38 MAP Kinase Assay Kit (New England
BioLabs). Specifically, total p38 MAP kinase was
immunoprecipitated using a p38 MAP kinase antibody and
incubated with GST-ATF-2 fusion protein in the presence
of ATP and kinase buffer. Phosphorylation of ATF-2 was
25 measured by Western blotting using a phospho-specific
ATF-2 antibody and ECL.

Statistical Analysis

The results for differences in mediator release
30 were analyzed for statistical significance by the

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unpaired Student's *t* test (two-tailed). All results are
expressed as the arithmetic mean ± SEM.

RESULTS

5   **Identification of a Differentially-Expressed mRNA in
    Mouse Mast Cells Stimulated with IgE and Antigen**
            To isolate genes involved in FceRI-mediated
    signaling, the C1.MC/C57.1 cell line, a cloned growth
    factor-independent mouse mast cell line of BALB/c origin
10 (Young *et al.*, *Proc. Natl. Acad. Sci. USA* 84:9175-9179
    (1987); Tsai *et al.*, *FASEB J.* 10, Abstract A1268
    (1996)), was used. It has been reported that
    C1.MC/C57.1 cells can exhibit many functional cellular
    responses characteristic of activated mast cells,
15 including the release of pre-formed mediators and
    cytokines upon FceRI receptor crosslinking (Gordon and
    Galli, *J. Exp. Med.* 174:103-107 (1991)). C1.MC/C57.1
    mast cells were sensitized with anti-DNP IgE mAb and
    challenged with $DNP_{30-40}HSA$ or vehicle alone for 30
20 minutes, 1 hour, or 2 hours. Total RNAs were extracted
    from the stimulated or unstimulated mast cells, and
    subjected to mRNA differential display analysis as
    described (Liang and Pardee, *Science* 257:967-971 (1992);
    Liang *et al.*, *Nucl. Acids Res.* 21:3269-3275 (1993)).
25   Twenty-six differential display experiments were
    performed using different combinations of 3' anchored
    primers and 5' arbitrary primers and different RNA
    isolates from various time intervals of stimulation.
    Total RNA was extracted from C1.MC/C57 mast cells that
30 had been activated by IgE and specific antigen for 1
    hour, and then amplified using $T_{12}MT$ anchored primers and

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the AP-5 arbitrary primer (5'-GTTGCGATCC) (SEQ ID NO: 7). Several differentially expressed bands were consistently detected, including band 60-4.

Band 60-4 was excised from the gel, reamplified,

5   and used as probe for subsequent Northern blot analysis. The 60-4 cDNA probe hybridized to a 2.8 kb mRNA whose expression was rapidly increased in C1.MC/C57.1 mouse mast cells that had been activated through the FcεRI for 30 minutes or 1 hour, but returned to baseline levels

10 after 2 hours. The 60-4 probe was subsequently subcloned, and sequence analysis revealed a 328 base pair cDNA with no significant homology to any other entry in the Genbank database.

15 **Cloning and Structure of Murine Rin2**

The 60-4 probe was used to screen a BALB/c mouse bone marrow-derived cultured mast cell (BMCMC) cDNA library constructed in the Uni-ZAP XR vector (Stratagene). One positive clone (SY-6) with a 1.1 kb

20 insert was isolated. Sequencing of the entire SY-6 partial cDNA clone revealed no significant homology with any known genes. To facilitate the cloning of the full-length cDNA, the SY-6 clone was used as a probe to assess the expression pattern of the identified novel

25 gene transcript by Northern blot analysis. The expression of this novel mRNA appeared widespread, as it was detected in many tissues, including the heart, liver, kidney, lung, skeletal muscle, testis spleen and brain. Because the identified gene transcript was

30 expressed fairly abundantly in the brain, the SY-6 partial cDNA clone was used to screen a mouse brain cDNA

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library (Stratagene) in an attempt to obtain the
full-length cDNA clone. Three positive clones (SY-A,
SY-B, SY-C) were identified after the tertiary
screening, and clone SY-A appeared to contain an insert
5 (2.6 kb) of the appropriate predicted size (2.8 kb).
        Sequencing of the entire SY-A clone (2664 base
pairs) revealed a potential translational initiation
codon (ATG) at base 60 and a stop codon (TGA) at base
1534. The predicted open reading frame (ORF) of 1476
10 base pairs encodes a predicted protein of 491 amino
acids with a molecular mass of 56.9 kDa (Figure 3). To
determine whether the SY-A cDNA could indeed express a
protein of the predicted size, the SY-A clone was
subcloned into a pGEM expression vector and subjected to
15 coupled *in vitro* transcription/translation in a wheat
germ lysate system. A specific product of an apparent
molecular mass of 60 kDa was detected. These results
are thus consistent with the size of the predicted
protein deduced from the predicted ORF. Moreover,
20 hydrophilicity plot (Kyte-Doolittle) analysis of the
predicted ORF revealed a highly hydrophilic protein with
a few short hydrophobic regions. Thus, the protein
product of the predicted ORF is likely to be a soluble
cytosolic protein.
25   The predicted amino acid sequence of SY-A cDNA was
used to search available protein databases using the
BLAST program network server. Over a particular stretch
of amino acid sequence, the SY-A gene product was found
to share significant sequence homology with members of a
30 putative GTPase-binding protein family such as Vps9p
(BLAST $P= 2.2e-19$), JC265 ($P= 5.2e-05$), and Rin1 ($P=
4.1e-05$) (Figure 4). Vps9p is a recently characterized

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yeast protein required for vacuolar protein sorting
(Burd et al., Mol. Cell. Biol. 16:2369-2377 (1996)).
The two mammalian proteins encoded by two closely
related human cDNAs, JC265 and Rin1, have been

5   identified as inhibitors of an activated Ras2 allele in
S. cerevisiae (Colicelli et al., Proc. Natl. Acad. Sci.
USA 88:2913-2917 (1991); Han and Colicelli, Mol. Cell.
Biol. 15:1318-1323 (1995)). Alignment of the amino acid
sequences of these proteins with that of SY-A showed

10   that the highest homology was found in the GTPase
binding homology (GBH) domain regions in which three GBH
motifs have been described (Burd et al., Mol. Cell.
Biol. 16:2369-2377 (1996)). Specifically, the SY-A
protein contains a 148-amino acid sequence which is 34%

15   identical (51/148) and 22% similar (33/148) to the
respective GBH domains of Vps9p containing the three GBH
motifs (Figure 4). Moreover, the SY-A gene product
contains an amino acid sequence (SADDFLPTL; SEQ ID NO:
8) that matches well with the proposed GBH motif II

20   (GADXFLPVL; SEQ ID NO: 9), and another downstream
sequence (GEDGYYFTN; SEQ ID NO: 10) that also aligns
well with the proposed GBH motif III (GEXXYYLTS; SEQ ID
NO: 11) (Burd et al., Mol. Cell. Biol. 16:2369-2377
(1996)) (Figure 4). Interestingly, the GBH motif II is

25   situated in a region of JC265 and Rin1 that has been
previously suggested to be distantly related to several
Ras-interacting proteins such as the human GAP, the
yeast IRA1 and IRA2 proteins, NF1, and sar1 (Colicelli
et al., Proc. Natl. Acad. Sci. USA 88:2913-2917 (1991)).

30   Because of its significant homology to the mammalian
protein Rin1, the protein encoded by the SY-A cDNA is

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herein called Rin2 (also called Rabex-5), and the gene
encoding the protein is herein called *rin2*.

Interation of Rin2 with Ras

5   Since Rin1 can suppress an activated Ras allele in
yeast and interact directly with yeast Ras2p and with
wild-type and activated human H-Ras (Han and Colicelli,
Mol. Cell. Biol. 15:1318-1323 (1995); Han, *Proc. Natl.
Acad. Sci. USA* 94:4954-4959 (1997)), it was of interest
10   to determine whether Rin2 could also interact directly
with Ras. Using the yeast two-hybrid system with the
entire open reading frame (residues 1-491) of murine
Rin2 expressed as a GAL4-activation domain fusion
protein and the wild-type murine H-Ras expressed as the
15   DNA-binding fusion protein, it was demonstrated that
murine Rin2 was able to interact with wild-type murine
H-Ras (Figure 12). Positive interactions with murine H-
Ras could also be detected for the N-terminal and the C-
terminal regions of Rin2, and for the region of Rin2
20   containing the three GBH domains (residues 203-380).
But the strength of interaction with murine H-Ras
protein, as measured by a quantitative β-galactosidase
assay, appeared to be the strongest for the full-length
murine Rin2 protein (Figure 12). However, unlike Rin1,
25   full length murine Rin2 proteins did not appear to bind
significantly to the wild-type yeast Ras2p, nor did any
of the three truncated fusion protein constructs
representing the three different regions of the Rin2
protein. These data indicate that Rin2 can interact
30   with Ras in ways that are distinct from those of Rin1.

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Decreased Expression of Rin2 Enhances Signaling
Responses Induced by FceRI Aggregation in Mast Cells

     The FceRI-induced activation of Ras in mast cells
results in an increase in transcriptional activity of
5   the transcription factor Elk-1 (Turner and Cantrell, *J.
Exp. Med.* 185:43-53 (1997)), which is a regulator of the
serum response element in the promoter region of
immediate early response genes such as c-*fos* (Treisman,
Curr. Opin. Genet. Dev. 4:96-101 (1994)). The
10 transcription activation of Elk-1 is mediated by the
Ras/Raf-1/MEK/Erk-MAP kinases pathway (Treisman, *Curr.
Opin. Cell Biol.* 8:205-215 (1996)). As described
herein, *rin2* mRNA was identified by virtue of its
enhanced expression in mast cells that are activated by
15 FceRI aggregation. Because Rin1 has been indicated as a
negative effector of Ras, the ability of Rin2 to
suppress the signaling responses initiated by Ras in
mouse mast cells activated by FceRI ligation was
assessed.

20   The SY-A cDNA was inserted into a CMV expression
vector (pBK-CMV) in the antisense orientation, and the
resulting plasmid (pBK-CMV-SYA-AS) was stably
transfected into mouse C1.MC/C57.1 mast cells. From two
independent transfections, eight separate stable lines
25 of antisense Rin2 transfectants and seven separate
stable lines of control CMV vector transfectants were
established.

     The G418-resistant colonies obtained were
stimulated with IgE and antigen, and the pattern of
30 expression of early response genes such as c-*fos* was
assessed using Northern blot analysis. Transfection of

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antisense *rin2* significantly enhanced the expression of
mRNA of c-*fos* in mouse mast cells activated by FceRI
crosslinking. In mouse mast cells transfected with the
control CMV plasmid, c-*fos* mRNA expression was rapidly

5   induced by 15 or 30 minutes after IgE and
antigen-dependent stimulation, but returned to
undetectable or basal levels by 1 hour after
stimulation. In contrast, in mouse mast cells
transfected with the *rin2* antisense construct, not only

10   was a significantly higher level of c-*fos* expression
observed at 15 or 30 minutes after IgE and
antigen-dependent stimulation, but a significant and
sustained induction of c-*fos* message was still
detectable in these activated mast cells at 1 hour after

15   stimulation.

        Similarly, the activation of Erk-MAP kinase
activity induced by FceRI activation was also greatly
potentiated in C1.MC/C57.1 mast cells that had been
stably transfected with the antisense *rin2* construct

20   (Figure 5). Consistent with results reported previously
(Tsai et al., *Eur. J. Immunol.* 23:3286-3291 (1993),
FceRI crosslinking caused a rapid activation of Erk-MAP
kinase activity in mouse mast cells which had been
transfected with the control plasmid 30 minutes after

25   stimulation (2.2 fold increase). The activation
persisted for 2 hours (approximately 1.5 fold increase)
and then declined to basal levels by 3 hours after
stimulation (1.0 fold). In contrast, in mouse mast
cells transfected with the *rin2* antisense plasmid, a

30   significantly higher level of Erk-MAP kinase activation
was induced by FceRI-dependent stimulation for 30
minutes (4.0 fold), 1 hour (3.6 fold), or 2 hours (2.2

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fold) after the addition of antigen, and such Erk-MAP
kinase activation was still sustained at 3 hours (1.3
fold).

    Stimulation of mast cells by FcεRI aggregation
5   leads to the activation of the Ras-mediated ERK-MAP
kinase, JNK, and p38 signaling pathways (Tsai et al.,
Eur. J. Immunol. 23:3286-3291 (1993); Hirasawa et al.,
J. Biol. Chem. 270:10960-10967 (1995); Zhang et al., J.
Biol. Chem. 272:13397-13402 (1997); Ishizuka et al.,
10 Proc. Natl. Acad. Sci. USA 94:6358-6363 (1997); Kawakami
et al., J. Immunol. 161:1795-1802 (1998)), and
activation of the ERK-MAP kinases and JNK in turn
regulate the synthesis and release of cytokines from
these cells. FcεRI crosslinking caused a rapid
15 activation of ERK-MAP kinase activity in control
transfected C1.MC/C57.1 cells by 30 minutes to 2 hours
after stimulation, which declined to basal levels by 3
hours after stimulation. In contrast, in the Rin2
antisense transfected mast cells, a significantly higher
20 level of ERK-MAP kinase activation was induced by FcεRI
stimulation 30 minutes to 2 hours after antigen
challenge, and high levels of ERK-MAP kinase activation
were still sustained at 3 hours. Antisense Rin2
expression also significantly potentiated the levels of
25 activation of JNK (Figure 14) and p38 MAP kinase (Figure
15) induced by FcεRI aggregation at 30 minutes to 3
hours after stimulation. Taken together, the results of
these studies indicate that reduced expression of Rin2
can result in marked potentiation of the Ras-mediated
30 signaling responses in mast cells, and suggest that Rin2
functions as a Ras inhibitor/effector.

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Decreased Expression of Rin2 Potentiates Release of Preformed Mediator from Mast Cells Activated by FcεRI Aggregation

Since reduced expression of Rin2 can potentiate
5   some of the intracellular signaling responses initiated
by FcεRI-dependent mast cell activation, the effect of
decreased Rin2 expression on the cells' secretory
responses to FcεRI-dependent activation was also
assessed.   C1.MC/C57.1 mast cells which had been
10 transfected with the rin2 antisense expression or
control vectors were stimulated with IgE and $DNP_{30-40}HSA$.
As shown in Figure 6, mouse mast cells stably
transfected with the rin2 antisense construct released
significantly higher levels of the preformed mediator,
15 5-HT, than did mast cells transfected with the pBK-CMV
vector only.   This effect was observed at all
concentrations of DNP antigen tested (1, 10, 50, or 100
ng/ml $DNP_{30-40}HSA$), indicating that the potentiating
effect of rin2 antisense expression was not antigen
20 dose-dependent.

Decreased Expression of Rin2 Potentiates Cytokine Release from Mast Cells Activated by FcεRI Aggregation

Crosslinking of FcεRI receptors not only induces
25 mast cells to release preformed mediators such as 5-HT,
but also induces these cells to secrete cytokines
(Gordon et al., Immunol. Today 11:458-464 (1990); Paul
et al., Adv. Immunol. 53:1-29 (1993)).   Thus, the effect
of changes in Rin2 expression on the FcεRI-dependent
30 release of IL-6 from mouse mast cells was also assessed.
As illustrated in Figure 7A, the release of IL-6 from

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mast cells 2, 4, or 6 hours after FceRI aggregation (50 ng/ml) was enhanced dramatically by the transfection of the cells with the *rin2* antisense expression vector, as compared with responses elicited in mast cells

5   transfected with the control CMV vector. This potentiating effect was more pronounced (greater than 20-fold increase) in cells which had been stimulated for longer time intervals (4-6 hours). Furthermore, when the transfected cells were stimulated by different

10   concentrations of $DNP_{30-40}HSA$ for 6 hours, mouse mast cells which had been transfected with the *rin2* antisense construct released significantly higher levels of IL-6 than did mast cells which had been transfected with the control vector at each concentration of antigen tested

15   (Figure 7B).

       As illustrated in Figure 13A, there were no significant differences between the amounts of β-hexosaminidase that were released from the antisense or control transfectants in response to FcεRI aggregation.

20   This result is consistent with findings that the release of pre-formed mediators can occur independently of signaling pathways involving Ras/MAP kinase (Hirasawa et al., *J. Biol. Chem.* *270*:10960-10967 (1995); Zhang et al., *J. Biol. Chem.* *272*:13397-13402 (1997)). On the

25   other hand, release of the cytokine IL-6 from the mast cell transfectants 6 hours after FcεRI aggregation was significantly enhanced (by a mean value of approximately 70%) in the antisense group (Figure 13A). The time course of this effect is shown in Figure 13B. In

30   general, those antisense transfectants which exhibited the largest and most sustained reduction in Rin2 protein

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expression after FcɛRI aggregation also exhibited the greatest enhancement of IL-6 production (Figure 13C).

Rin2 mRNA Expression is Up-regulated in Mouse Mast Cells
5 Stimulated with Stem Cell Factor and in PC12 Cells
Stimulated with Nerve Growth Factor

To assess whether the enhanced expression of the *rin2* transcript was specific for the FcɛRI-dependent signaling pathway in mast cells, Northern analysis was
10 performed to examine the expression of *rin2* mRNA in mouse bone marrow derived cultured mast cells (BMCMC) which had been stimulated with the growth factor, stem cell factor (SCF), the ligand for the c-kit receptor tyrosine kinase (Galli *et al.*, *Adv. Immunol.* 55:1-96
15 (1994)). The widespread tissue distribution of *rin2* mRNA suggests that Rin2 may function as a signaling element in the functional responses of many cell types besides mast cells. Therefore, *rin2* expression was also analyzed in PC12 cells, an adrenal pheochromocytoma cell
20 line, which had been stimulated with nerve growth factor (NGF) (Greene and Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428 (1976)).

rin2 mRNA expression was rapidly increased by 30 minutes to 1 hour after stimulation of BMCMCs with SCF
25 (50 ng/ml), but returned nearly to baseline levels after 2 hours. Expression of the *rin2* transcript was also significantly induced 30 minutes after NGF stimulation (50 ng/ml) in PC12 cells, and the enhanced expression of the transcript was still detectable 2 hours after
30 stimulation. Thus, SCF induced a transient enhancement of *rin2* mRNA expression in mast cells, but NGF elicited

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a much more sustained activation of *rin2* expression in
PC12 adrenal pheochromocytoma cells.

Decreased Expression of *Rin2* Enhances Cell Proliferation
5 **in PC12 Cells**

      PC12 cells stimulated with NGF undergo neuronal
differentiation, which is characterized by the extension
of neurites and the cessation of cell proliferation
(Greene and Tischler, *Proc. Natl. Acad. Sci. USA*
10 *73*:2424-2428 (1976)). Since NGF promoted a sustained
induction of *rin2* mRNA expression in PC12 cells, the
effects of reduced *rin2* expression on cell proliferation
rate and NGF-induced neurite outgrowth were examined in
these cells. The antisense expression vector pBK-CMV-
15 SYA-AS was stably transfected into PC12 cells, and the
transfected cells were plated onto collagen-coated
plates and stimulated with NGF at 25, 50 or 100 ng/ml.
After 3-5 days, neurite outgrowth was observed both in
PC12 cells that had been transfected with the antisense
20 expression vector and in those cells that had been
transfected with the control CMV vector. No detectable
differences in the time of onset or the number of
neurite-bearing cells could be observed between these
populations of transfected cells. These results thus
25 indicate that Rin2 may not be directly involved with
NGF-induced neuronal differentiation in PC12 cells. On
the other hand, PC12 cells which had been transfected
with the antisense expression vector and then maintained
in their normal culture medium exhibited a significantly
30 higher rate of cell proliferation, as measured by a ^{3}H-
thymidine assay, than did the cells which had been

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transfected with the control pBK-CMV vector (Figure 8A).
This effect was also observed when the transfected PC12
cells were stimulated with NGF (100 ng/ml) for 18 hours
in medium containing 0.5% FCS (Figure 8B). Since NGF is

5 known to be anti-mitogenic to PC12 cells (Greene and
Tischler, *Proc. Natl. Acad. Sci. USA* 73:2424-2428
(1976)), these results are consistent with the
hypothesis that Rin2 expression results in the
inhibition of cell proliferation in PC12 cells and

10 perhaps other neuronal cells.

**Rin2 mRNA Expression is Up-Regulated in EL-4 T Cells
Stimulated via the T Cell Receptor Complex**
     The binding of peptide antigen and major

15 histocompatibility complex molecules to the T cell
receptor (TCR) induces T cell activation, which includes
the secretion of effector cytokines by the T cells and
the induction of mitotic activity in the T cells
themselves. Extensive evidence has shown that the

20 activation of Ras is involved in some of the
intracellular signaling pathways mediating T cell
activation (Lowy and Willumsen, *Ann. Rev. Biochem.*
62:851-891 (1993)). To assess whether T cell activation
could result in enhanced expression of the *rin2*

25 transcript, Northern analysis was performed to examine
the expression of *rin2* mRNA in EL-4 mouse T cells that
had been stimulated via the TCR by incubating the cells
with both anti-CD3 antibody (1 μg/ml) and anti-CD28
antibody (2 μg/ml). *rin2* mRNA expression was rapidly

30 increased by 30 minutes to 1 hour after TCR stimulation,
but declined nearly to baseline levels after 2 hours.

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The time course of this effect on *rin2* mRNA expression
in T cells is thus similar to that observed in mouse
mast cells stimulated with SCF or IgE and antigen.

5   EQUIVALENTS

Those skilled in the art will recognize, or be able
to ascertain, using no more than routine
experimentation, many equivalents to the specific
embodiments of the invention described herein. Such
10 equivalents are intended to be encompassed by the
following claims:

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CLAIMS

We claim:

1. An isolated Rin2 polypeptide which down-regulates
    functional responses elicited by Ras-dependent
    signaling pathways, or a derivative or portion
    thereof having Rin2 activity.

2. An isolated Rin2 polypeptide according to Claim 1,
    wherein the Ras-dependent signaling pathway is
    FceRI aggregation.

3. An isolated Rin2 polypeptide according to Claim 1,
    wherein said Rin2 polypeptide comprises the amino
    acid sequence of SEQ ID NO: 2.

4. An isolated Rin2 polypeptide according to Claim 1,
    wherein said Rin2 polypeptide has the same amino
    acid sequence as endogenous Rin2.

5. An isolated Rin2 polypeptide according to Claim 1,
    wherein said Rin2 polypeptide comprises an amino
    acid sequence which is at least about 40% similar
    to the amino acid sequence of SEQ ID NO: 2.

6. An isolated nucleic acid molecule which encodes a
    Rin2 polypeptide which down-regulates functional
    responses elicited by Ras-dependent signaling
    pathways, or a derivative or portion thereof having
    Rin2 activity.

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7.   An isolated nucleic acid molecule according to
      Claim 6, wherein said nucleic acid molecule
      comprises the nucleotide sequence of SEQ ID NO: 1
      or the complement of SEQ ID NO: 1, or a derivative
5   or portion thereof.

8.   An isolated polypeptide encoded by a nucleic acid
      molecule according to Claim 7.

10 9.   An isolated nucleic acid molecule according to
      Claim 6, wherein said nucleic acid molecule
      comprises one or more nucleotide sequences selected
      from the group consisting of:
      a)   SEQ ID NO: 12 or the complement of ID NO: 12;
15   b)   SEQ ID NO: 13 or the complement of SEQ ID
            NO: 13;
      c)   SEQ ID NO: 14 or the complement of SEQ ID NO:
            14;
      d)   SEQ ID NO: 15 or the complement of SEQ ID NO:
20   15; and
      e)   derivatives or portions of (a)-(d).

10.   An isolated protein encoded by an isolated nucleic
      acid molecule according to Claim 8.
25

11.   An isolated nucleic acid molecule according to
      Claim 6 comprising a nucleotide sequence which
      selectively hybridizes to all or a portion of SEQ
      ID NO: 1.
30

12.   An isolated nucleic acid molecule according to
      Claim 6 comprising a nucleotide sequence which

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selectively hybridizes to all or a portion of one
or more nucleotide sequences selected from the
group consisting of SEQ ID NO: 12, SEQ ID NO: 13,
SEQ ID NO: 14 and SEQ ID NO: 15.

5

13. An isolated nucleic acid molecule according to
     Claim 6 comprising a nucleotide sequence which is
     at least about 75% identical to SEQ ID NO: 1.

10 14. An isolated nucleic acid molecule according to
     Claim 13 comprising a nucleotide sequence which is
     at least about 90% identical to SEQ ID NO: 1.

15. An isolated nucleic acid molecule according to
15   Claim 6 comprising a nucleotide sequence which is
     at least about 75% identical to one or more
     nucleotide sequences selected from the group
     consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID
     NO: 14 and SEQ ID NO: 15.

20

16. An isolated nucleic acid molecule according to
     Claim 15 comprising a nucleotide sequence which is
     at least about 90% identical to one or more
     nucleotide sequences selected from the group
25   consisting of SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID
     NO: 14 and SEQ ID NO: 15.

17. A DNA construct comprising an isolated nucleic acid
     molecule according to Claim 6 operatively linked to
30   a regulatory sequence.

18. A recombinant host cell comprising an isolated
nucleic acid molecule according to Claim 6
operatively linked to a regulatory sequence.

5   19. A method for preparing a Rin2 polypeptide,
comprising culturing the recombinant host cell of
Claim 18.

20. An antibody, or an antigen-binding fragment
10   thereof, which selectively binds to the Rin2
polypeptide according to Claim 1, or a derivative
or portion thereof having Rin2 activity.

21. A method for assaying the presence of a Rin2
15   polypeptide according to Claim 1 in a cell or cell
lysate, comprising contacting said cell or cell
lysate with an antibody which specifically binds to
the Rin2 polypeptide.

20 22. A method of identifying an agent which alters
activity of a Rin2 polypeptide according to Claim
1, comprising the steps of:
(a) contacting a cell containing the Rin2
polypeptide, or a derivative or portion
25   thereof having Rin2 activity, with a stimulus
which activates at least one Ras-dependent
pathway in said cell, in the presence of the
agent to be tested; and
(b) identifying alteration of Rin2 activity.
30

23. A method according to Claim 22, wherein the
stimulus is selected from the group consisting of

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nerve growth factor, stem cell factor, peptide
antigen and major histocompatibility complex
molecules, and IgE and specific antigen.

5   24.   A novel agent which alters Rin2 activity identified
according to the method of Claim 23.

25.   A method of enhancing activity of a Rin2
polypeptide according to Claim 1, comprising
10   contacting said Rin2 polypeptide, or a cell
containing said Rin2 polypeptide, with an agent
that enhances the activity of Rin2.

26.   A method of inhibiting a functional response
15   induced by a Ras-dependent signaling pathway in a
cell, comprising contacting said cell with an agent
which enhances the activity of a Rin2 polypeptide.

27.   A method according to Claim 26, wherein the cell
20   expresses a receptor having a signaling pathway
regulated by Rin2.

28.   A method according to Claim 27, wherein the
receptor is selected from the group consisting of
25   FcεRI, TrkA, c-kit and T cell receptor.

29.   A method according to Claim 26, wherein the
functional response is one or more responses
selected from the group consisting of activation of
30   ERK-MAP kinase, activation of JNK kinase and
activation of p38 MAP kinase.

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30. A method according to Claim 26, wherein the functional response is a cellular secretory response.

5 31. A method according to Claim 30, wherein the cellular secretory response is release of preformed mediators, lipid mediators and/or cytokines.

32. A method of inhibiting a Ras-dependent signaling
10 pathway in a cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.

33. A method of inhibiting cellular proliferation in a
15 cell, comprising contacting said cell with an agent which enhances the activity of a Rin2 polypeptide.

34. A method according to Claim 33, wherein the cell expresses the TrkA receptor for nerve growth
20 factor.

35. A method of inhibiting Ras-dependent signaling in a mammal comprising administering to the mammal an effective amount of an agent which enhances the
25 activity of Rin2.

36. A method of inhibiting a functional response induced by a Ras-dependent signaling pathway in a mammal, comprising administering to the mammal an
30 effective amount of an agent which enhances the activity of Rin2.

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37.   A method of inhibiting Ras-dependent signaling in
      the mast cells of a mammal, comprising
      administering to the mammal an effective amount of
      an agent which enhances the activity of Rin2.

5

38.   A method of inhibiting Ras-dependent signaling in T
      cells of a mammal, comprising administering to the
      mammal an effective amount of an agent which
      enhances the activity of Rin2.

10

39.   A method of inhibiting Ras-dependent cell
      proliferation in a mammal, comprising administering
      to the mammal an effective amount of an agent which
      enhances the activity of Rin2.

15

40.   A method of treating an allergic condition in a
      mammal comprising administering to the mammal an
      effective amount of an agent which enhances the
      activity of the Rin2 polypeptide.

20

41.   A method according to Claim 40, wherein the
      allergic condition is asthma, hay fever or atopic
      eczema.

25 42.   A method of treating a disorder selected from the
      group consisting of Ras-dependent cancer, Ras-
      dependent neoplastic cellular proliferation and
      Ras-dependent unwanted non-neoplastic cellular
      proliferation in a mammal, comprising administering

30   to the mammal an effective amount of an agent which
      enhances the activity of a Rin2 polypeptide.

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43.   A method of inhibiting functional responses induced
       by T cell receptor-dependent activation of T cells
       in a mammal comprising administering to the mammal
       an effective amount of an agent which enhances the
5   activity of Rin2.

44.   A method of treating a disorder selected from the
       group consisting of autoimmune disorder, T cell-
       associated disorder, T cell-dependent graft
10   rejection and graft-versus-host disease in a
       mammal, comprising administering to the mammal an
       effective amount of an agent which enhances the
       activity of a Rin2 polypeptide.

15 45. A method according to Claim 44, wherein the
       disorder is selected from the group consisting of
       autoimmune (Type I) diabetes mellitus, rheumatoid
       arthritis, ankylosing spondylitis, sarcoidosis,
       Sjögren's syndrome, multiple sclerosis,
20   inflammatory bowel disease, Crohn's disease,
       ulcerative colitis, dermatomyositis, scleroderma,
       polymyositis, systemic lupus erythematosus, biliary
       cirrhosis, autoimmune thyroiditis, autoimmune
       hepatitis, psoriasis, contact sensitivity and
25   atopic dermatitis.

46.   A method of inhibiting activity of a Rin2
       polypeptide according to Claim 1, comprising
       contacting said Rin2 polypeptide, or a cell
30   containing said Rin2 polypeptide, with an agent
       that inhibits the activity of Rin2.

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47. A method of enhancing a functional response induced by a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which inhibits the activity of a Rin2 polypeptide.

48. A method according to Claim 47, wherein the cell expresses a receptor having a signaling pathway regulated by Rin2.

49. A method according to Claim 48, wherein the receptor is selected from the group consisting of FceRI, TrkA, c-kit and T cell receptor.

50. A method of enhancing a Ras-dependent signaling pathway in a cell, comprising contacting said cell with an agent which inhibits the activity of a Rin2 polypeptide.

51. A method of enhancing a functional response induced by a Ras-dependent signaling pathway in a mammal, comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

52. A method of enhancing Ras-dependent signaling in a mammal comprising administering to the mammal an effective amount of an agent which inhibits the activity of Rin2.

53. A method of enhancing functional responses induced by T cell receptor-dependent activation of T cells in a mammal comprising administering to the mammal

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an effective amount of an agent which inhibits the
activity of Rin2.

54. A method of enhancing Ras-dependent signaling in
the mast cells of a mammal, wherein the desired
outcome is increased mast cell function, comprising
administering to the mammal an effective amount of
an agent which inhibits the activity of Rin2.

55. A method of enhancing Ras-dependent signaling in T
cells of a mammal, wherein the desired outcome is
increased T cell function, comprising administering
to the mammal an effective amount of an agent which
inhibits the activity of Rin2.

56. A method of enhancing Ras-dependent cell
proliferation in a mammal, comprising administering
to the mammal an effective amount of an agent which
inhibits the activity of Rin2.

57. A method according to Claim 56, which produces
enhanced wound healing, angiogenesis, and/or re-
epithelialization.

FIG. 1

Normal Rin2 Expression/Function

Rin2 → (−) Active Ras → Normal Ras-Dependent Signaling → Normal Responses

Enhancement of Rin2 Expression/Function

Rin2 → (−) Active Ras → Decreased Ras-Dependent Signaling → Decreased Responses

Inhibition of Rin2 Expression/Function

Rin2 → (−) Active Ras → Increased Ras-Dependent Signaling → Increased Responses

Examples of normal responses regulated by Ras-dependent signaling	Settings in which it is desirable to decrease such responses	Settings in which it is desirable to increase such responses
Mast cell mediator production	Asthma, hay fever, atopic dermatitis	Immunity to pathogens
T cell function	Graft rejection; T cell dependent diseases (certain autoimmune diseases, contact dermatitis, graft-versus-host disease)	HIV infection; certain other infections; immunosupressions associated with cancer therapies
Cell proliferation	Cancer; proliferation of vascular wall cells in arteriosclerosis	Wound healing; angiogenesis (e.g. to improve ischemia; re-epithelialization); nerve regeneration

FIG. 2

```
     GCACGAGGGGCGCTGGGAGCGCCGGTCGGAGCGCCGCGGAGCAGGAAGAAG ATG                         62
                                                         Met                         1

 1   AGC CTG AAG TCC GAA CGC AGG GGA ATT CAT GTG GAT CAA TCT GAG CTC CTG TGC AAG      122
     Ser Leu Lys Ser Glu Arg Arg Gly Ile His Val Asp Gln Ser Glu Leu Leu Cys Lys     21

63   GGA TGC TAC TAC GGT TTC TGC AAG TCC TGG AGG                                      182
     Gly Cys Tyr Tyr Gly Phe Cys Lys Ser Trp Arg                                     41

 2   GAG TAC AAG GCC AGG AAC GGC TAC GGT Phe TGG Cys                                  242
     Glu Tyr Lys Ala Arg Asn Gly Tyr Gly Phe Trp Cys                                 61

123  GAG CAC CGG CGG CAG ATC GAC GAC GCA CTG                                         302
     Glu His Arg Arg Gln Ile Asp Asp Ala Leu                                         81

22   Tyr His Lys Arg Ala Arg Gln Glu Ala Trp                                         362
                                                                                     101

183  Glu Glu Glu Gly Glu Ala Glu Gly Ser Gln                                         422
                                                                                     121

42   Gln Arg Glu Glu Gly Ala Gly Gln Ala Ser                                         482
                                                                                     141

243  Gln Arg Glu Glu Ala Phe Ala Gln Gly Leu                                         542
                                                                                     161
```

FIG. 3A

```
1023  ATC GTC CTG AAG GGC AAC CCC CCT CGC CTG CAG TCC AAC ATC CAG TAC ATC ACT CGC TTC  1082
 322  Ile Val Leu Lys Gly Asn Pro Pro Arg Leu Gln Ser Asn Ile Gln Tyr Ile Thr Arg Phe   341

1083  TGC AAC AGC CTC ATG GGC GAT GAG TAC TTC AAT TTA AGT CTC TAC AAC CTG TAC AAC TGC TGT  1142
 342  Cys Asn Ser Leu Met Gly Asp Glu Tyr Phe Asn Leu Ser Leu Tyr Asn Leu Tyr Asn Cys   361

1143  GCT GTG GCT TTC ATT GAA AAA TCT GCC CAG TAT CTT GAG CAG TTG AGT CAG TTT TTG GAG GAT  1202
 362  Ala Val Ala Phe Ile Glu Lys Ser Ala Gln Tyr Leu Glu Gln Leu Ser Gln Phe Leu Glu Asp  381

1203  GAC CGG TAC ATG TCT GGC CAG ACA TCC CCC AGG AAG CAG GAG AGT GAG TGG CCC CCG  1262
 382  Asp Arg Tyr Met Ser Gly Gln Thr Ser Pro Arg Lys Gln Glu Ser Glu Trp Pro Pro   401

1263  GAG GCC TGC TTA GGT GTG CAA ATG TAT AAG AAC TTG GAC CTC TCT CAG TTG AAT  1322
 402  Glu Ala Cys Leu Gly Val Gln Met Tyr Lys Asn Leu Asp Leu Ser Gln Leu Asn   421

1323  GAA CGG CAA GAA AGG ATC ATG AAC CTT GAA CTT GAG TTA ATA GAC TGG  1382
 422  Glu Arg Gln Glu Arg Ile Met Asn Leu Glu Leu Glu Leu Ile Asp Trp   441

1383  ACA GAC GGG ATT GCC AAG GTT CAA GAC AAA TAC CCA CTG GAG ATT AAG  1442
 442  Thr Asp Gly Ile Ala Lys Val Gln Asp Lys Tyr Pro Leu Glu Ile Lys   461

1443  CCC CCG AAC CAA CCC TTA GCA GCC ATC GAC TCT GAG AAC GAC AAG CTC CCT  1502
 462  Pro Pro Asn Gln Pro Leu Ala Ala Ile Asp Ser Glu Asn Asp Lys Leu Pro   481

1503  CCC CCT CTG CAG CCT GTG TAC GCA GGG TGA CGG CCC CTG TTA TTT GGG GCC TGG TTC TGG GAG CTG  1571
 482  Pro Pro Leu Gln Pro Val Tyr Ala Gly ***   491
```

```
1572  CTGCGTTCCACTGTTCAGGTCCGGAATATGAACTGACTGCTTAAAGTTTCAAAGTGTTTTAGGTACAGATTTAGGGAT  1650
1651  TGGTTATTCTCTTTTTCTTCTGTCGCTCTTTTATGTGTGTAATAATAATGTACTATTTATTTGAGCTGGTGAGTAGG  1729
1730  TTTGTGTGAATTCGTCTCTTTTATGTGTGCACTGCCTCTTTATGCCCTGATTCCCATGGCTTCCTCTGTGTAGACACTGTTGTTGGT  1808
1809  TTTGGGCAAACACTGCCTTTTAAAGGATAAAAACAGATCTATAAAGTGTTGAAATGAATAAGTAATATGTAAAATATGT  1887
1888  CCCCCAGTGTGAAATAATTTGTAATTGTAAAGATAAGATAATGTAAGAAGATAATATGTAAAATTGTAAATATGT  1966
1967  AAAAAAAAAAACACATAGGGCTGGGGAGGGGCTGTCTCAGCGTGCATGCCATTTTATATGGTTTGTCCCACTAAAAGAATCTAAAGA  2045
2046  TAGGTGAAAATGAAATTTATTGAATGTTTGCACTAACTGCCCTAACTGCTCTTTCTGAGATTCTTTCTGGTACATGTAAAGTTGTAACGC  2124
2125  ATTTGAGCTTTAACAGGACATTGCACAGAATTATTCTGGGGACCCCGGTGCTCAGTCAGTAAAGTGCTCTCCTATACTGAT  2203
2204  CAACTTTTAGGTCACATAGCAGTCCCTGTCCGCCCCGGTGGGCATCGTGTCAATGAGAGGGCATCGTGTCAATGAGAGGGC  2282
2283  GTGAGGACCTGAGCTCCTGGGGCAGGGGACGTCCTGGGGCAGGGGATGCCCTCCTGCGCTGCCTCTGGCACCTCGTTGAAAGCTGGGCATGTCGTGGA  2361
2362  GCCAGAGTTCCTGGGGCAGGGGATGCCCTCCTGCGCTGCCTCTGGCACCTCGTTGAAAGCTGGGCATGTCGTGGA  2440
2441  GAGAAATGATAGCCAAGAGACACACACAGTGCCCATCGTCAATGAGAGAGCCCACATGTGAACTGGAACTTGACTTGGAACTTGACTCTGTAGC  2519
2520  ACACACACCATGAACACACAGTTGCTGATTAGTAGTACACACAGTTGCTGTCCAGCTTCCTTTCCCTGTTATCC  2598
2599  AATAAACTTCCCCCACAGTGCTGTGGGGCTATTGCCTTTTTATCTGAAAAAAAAAAAAAAAA  2664
```

FIG. 3B

Rin2   227   ETTDDEKKDL   AIQKRIRALH   WVTPQMLCVP   VNEEIPEVSD   MVVKAITDII
Vps9p 157   EHMKDLTNDD   TLLEKIRHYR   FISPIMLDIP   DTMPNARLNK   FVHLASKELG
Rin1   155   RLAADG-SLG   RLAEGLRLAR   AQGPGAFGSH   LSLPSPVE--   -LEQVRQKLL
JC265 182   FHMADG-SWK   QLKENLQLVR   QRNPQELGVF   APTPDFVD--   -VEKIKVKFM

                                                     GAD FL PVL

                                                     MOTIF II

Rin2   277   EMDSKRVPRD   KLACITRCSK   HIFNAIKITK   NEPASADDFL   PTLIYIVLKG
Vps9p 207   KINRFKSPRD   KMVCVLNASK   VIFGLLKHTK   LEQNGADSFI   PVLIYCILKG
Rin1   201   QLVRTYSPSA   QVKRLLQACK   LLYMALRTQE   GEGSGADGFL   PLLSLVLAHC
JC265 228   TMQKMYSPEK   KVMLLLRVCK   LIYTVMENNS   GRMYGADDFL   PVLTYVIAQC

             LL CK LIY

             MOTIF I

Rin2   327   NPPRLQSNIQ   YITRFCNPSR   LMTGEDGYYF   TNLCCAVAFI   EKLDAQSL
Vps9p 257   QVRYLVSNVN   YIERFRSPDF   I-RGEEEYYL   SSLQAALNFI   MSLTERSL
Rin1   251   DLPELLLEAE   YMSELLEPSL   L-TGEGGYYL   TSLSASLALL   SGLGQAHT
JC265 278   DMLELDTEIE   YMMELLDPSL   L-HGEGGYYL   TSAYGALSLI   KNFQEEQA

                                     GE YYL TS

   MOTIF III

FIG. 4
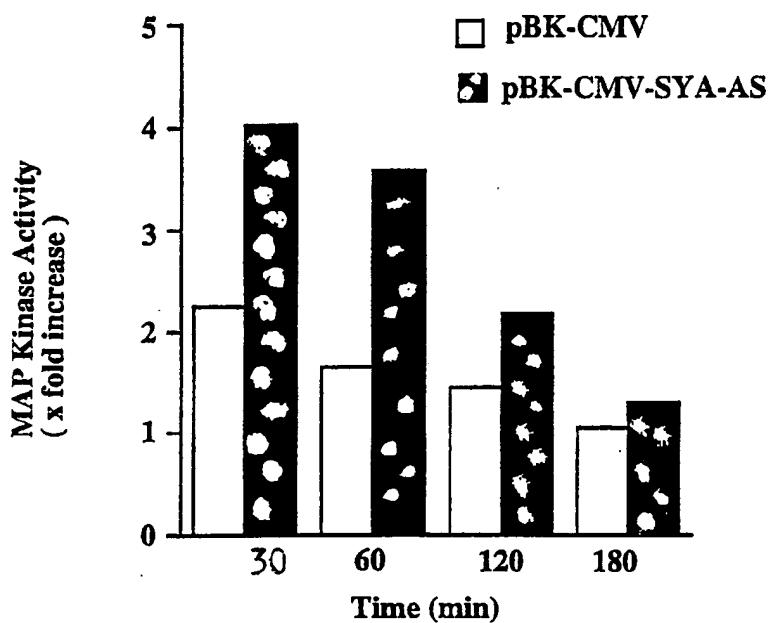
FIG. 5
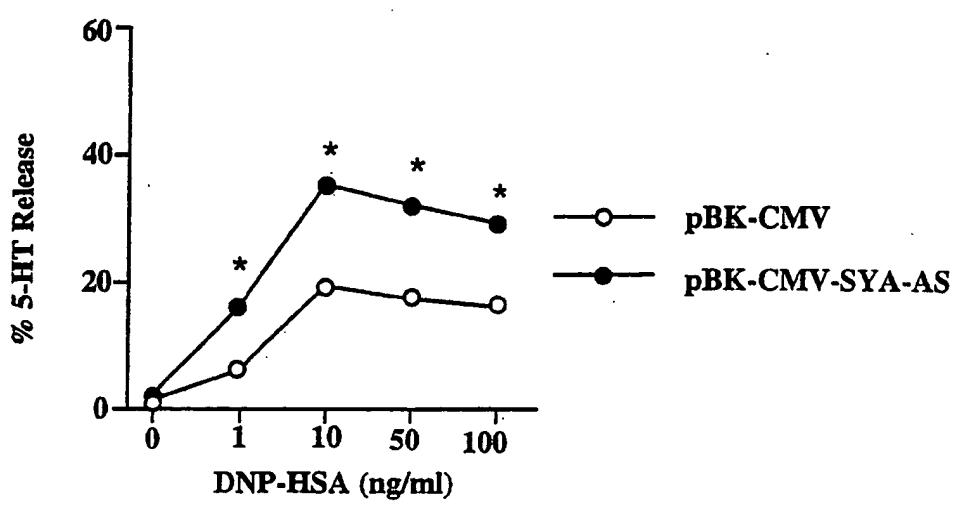
FIG. 6
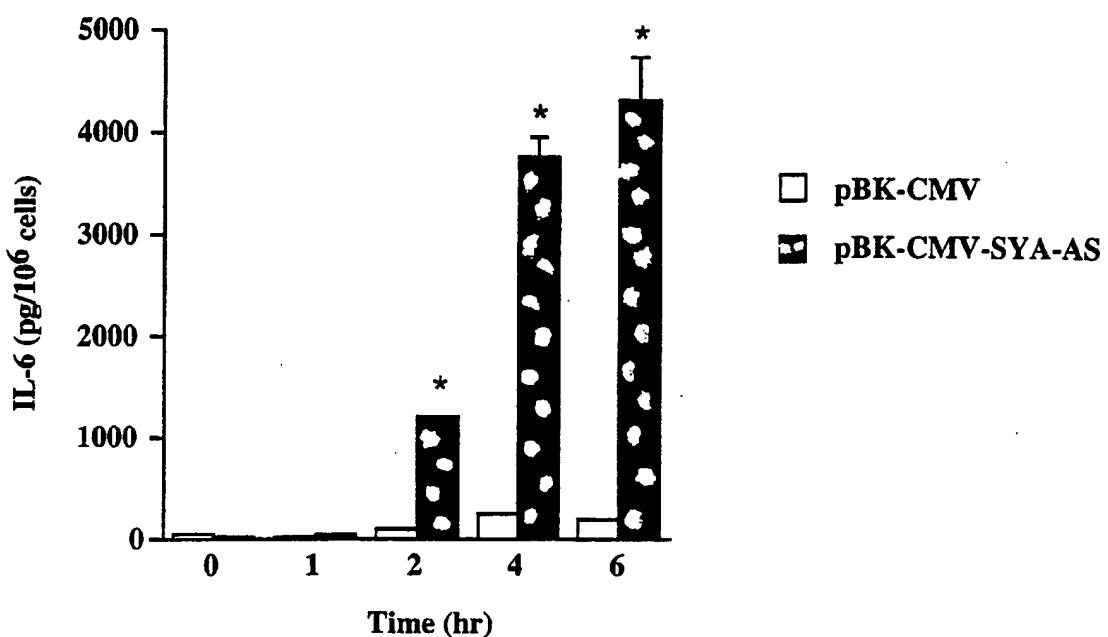
FIG. 7A
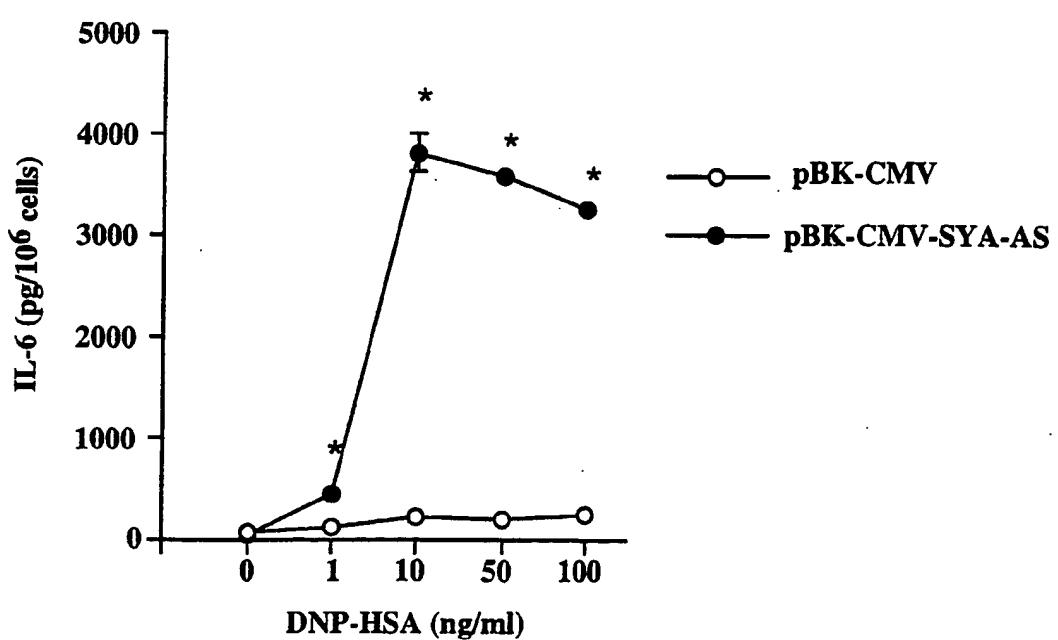
FIG. 7B
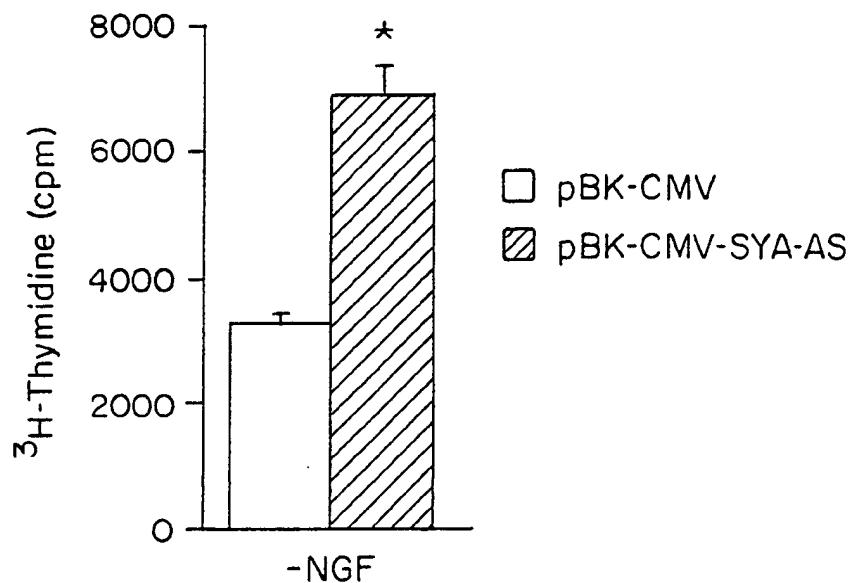
FIG. 8A
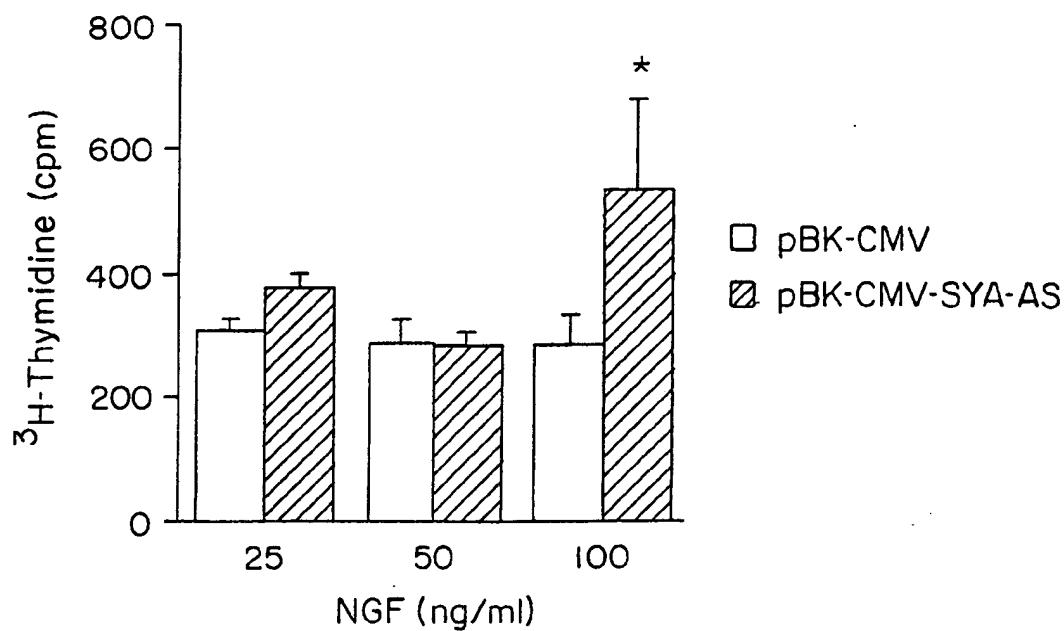
FIG. 8B

HRIN2-1B1=1.3kb
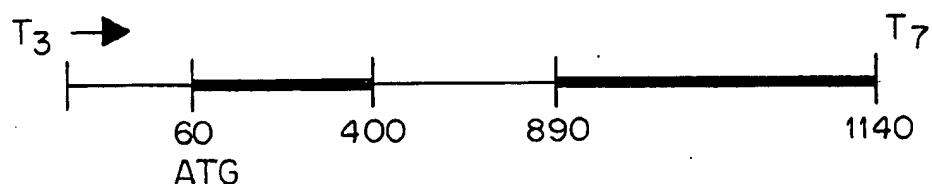
HRIN2-2B1=2.9kb
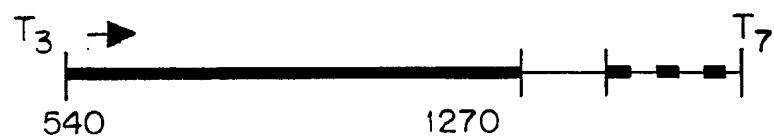
number=nt # in murine Rin2 sequence

■--■--■- 3' end sequenced

████ Coding region sequenced

FIG. 9

79.2% identity in 355 nt overlap; score: 883
Murine Rin2
        60   70   80   90   100   110
      AGGAAGAAGATGAGCCTGAAGTCCGAACGCAGGGGAATTCATGTGGATCAATCTGAGCTC
      : ::::::::::::::::: : ::: :::::: : :::::::::::::::
      AAGAAGAAGATGAGCCTTAGGTCTGAACGCCGAGGAATTCATGTGGATC-----------

HRIN2-1B1
        120   130   140   150   160   170
      CTGTGCAAGAAAGGATGCGGTTACTACGGCAACCCTGCCTGGCAGGGTTTCTGCTCCAAG
      :::::::::::::: ::::::: ::::::::: :::::::::::::::::::::::::::::
      CTGTGCAAGAAAGGATGTGGTTACTGTGGCAACCCTACCTGGCAGGGTTTCTGCTCCAAG

        180   190   200   210   220   230
      TGCTGGAGGGAGGAGTACCACAAGGCCCGGCAGAGGCAGATCCAAGAGGACTGGGAACTG
      :::::::::::: ::::: ::::: ::: :::::: :::::: :: ::: ::::::: :::
      TGCTGGAGGGAAGAGTAGCACAAAGCCAGGCAGAAGCAGATTCAGGAGTACTGGGAGCTG

        240   250   260   270   280   290
      GCAGAACGACTTCAGCGGGAGGAGGAAGAGGCCTTCGCGAGCAGCCAGAGCAGCCAACGA
      : :::::::: :..::::::::: :::::: :::: :: :.::: :::::::::::::::
      GTGGAACGACTCCNNCGGGAGGAAGAAGAG-CCTTTGCCANCAGTCAGAGCAGCCAAGGG

        300   310   320   330   340   350
      GCCCAGTCCCTCACCTTCTCCAAGTTCGAGGAGAAGAAGACCAATGAGAAAACCCGAAAA
      ::::: ::::::: ::::::::. :: :: : ::::: ::::: .::::. ::::. ..
      GCCCAATCCCTCATATTCTCCANCTTTGAAGGAAAGAAAACCAACNAGANCACCCNCNNG

        360   370   380   390   400
      GTCACCACAGTGAAGAAGTTCTTCAGCGCCTCTTCCAGAGCTGGATCCAAGAAGG
      :: ::::::::::::.:: : ::::::: : :::::::::: : ::::: :: :: :
      GTTACCACAGTGAANAAAT-CTTCAGTACGTCTTCCAGGGTCGGATCAAAAAAAAG

FIG. 10A

```
      78.6% identity in 266 nt overlap; score:  607
Murine Rin2
            890         900         910         920         930         940
      ATTGAGATGGACT--CAAAGCGTGTGCCTCGGGACAAGCTGGCC-TGCATCACC-AGGTG
      :::::  :::::  :   ::   :  ::::::.:::  ::  :::::::::::  :::.::::: :  :  :
HRIN2-1B1
      ATTGAAATGGATTNCCAGGGGGTGTGCNTCGAGAAAAGCTGGCCNTGCNTCACCCAAGGG


            950         960         970         980         990         1000
      CAGCAAGCACATCTTCAATGCCATCAAGATCACCAAGAATGAGCCAGCCTCTGCCGATGA
      ::::::::.:.:: ::. :.::::::::::::::::::  :::: ::::  ::  :: ::  :::::
      CAGCAAGNANATTTTTNGANGCCATCAAGATCACTTAGAACGAGCTGGCGTCAGCAGATGA


            1010        1020        1030        1040        1050
      CTTCCTGCCC-ACCCTGATCTACATCGTCCTGAAGGGCAACCCCCCTCGCCTGCAGTCCA
      .::  ::  :::  :::::  ::  :::::  ::   :::::::::::::::::  :  :::::  ::::     :
      NTTTCTTCCCCACCCTCATTTACATTGTTTTGAAGGGCAACCCCCAT-GCCTTCAGTTTA


      1060        1070        1080        1090        1100        1110
      ACATCCAGTACATCACTCGCTTCTGCAACCCCAGCCGGCTCATGACGGGCGAGGATGGCT
      :  :::::::::  :::::  :::::::::::::::  ::  :::::  ::  :::::  ::  ::::::::::::
      ATATCCAGTATATCACGCGCTTCTGCAATCCAAGCCGACTGATGACTGGAGAGGATGGCT


      1120        1130        1140
      ACTACTTCACCAACCTGTG-CTGTGC
      ::::  :::::::::  :::  :  :::  ::
      ACTATTTCACCAATCTGAGGCTGGGC
```

FIG. 10B

83.5% identity in 746 nt overlap; score: 2027

```
Murine Rin2 540        550        560        570        580        590
           AACAGACGAAGATGTTTTTGGAAGCAATGCCCTTATAAAAGGGATTTAAGCATCGAGGAAC
           : ::::: ::: :::::::::::::: ::::: ::: ::::::::: :::::::: :: ::::
           AGCAGACCAAGCTGTTTTTGGAAGGAATGCATTACAAAAGGGATCTAAGCATTGAAGAAC
HRIN2-2B1


           600        610        620        630        640        650
           AGTCAGAATGTACTCAGGACTTTTTACCAAAATGTGGCTGAAAGAATGCAGACCCGTGGGA
           ::::::::: ::: :::::::: :: ::::: ::::::::: ::::: ::::: :: :::::::
           AGTCAGAGTGTGCTCAGGATTTCTACCACAATGTGGCCGAAAGGATGCAAACTCGTGGGA


           660        670        680        690        700        710
           AAGTGCCTCCAGAGAAAGTGGAGAAGATAATGGATCAGATCGAAAAGCACATCATGACGC
           :::::::::::::: : ::: :::::::::::::::::::::: :::::: ::::::::::: :
           AAGTGCCTCCAGAAAGAGTCGAGAAGATAATGGATCAGATTGAAAAGTACATCATGACTC


           720        730        740        750        760        770
           GTCTCTATAAATTTGTGTTCTGCCCAGAGACTACTGATGATGAGAAGAAAGATCTCGCCA
           ::::::::::::: ::: ::::: ::::: ::::::::::::::::::::::::::::: ::::
           GTCTCTATAAATATGTATTCTGTCCAGAAACTACTGATGATGAGAAGAAAGATCTTGCCA


           780        790        800        810        820        830
           TTCAAAAAAGAATCAGGGCCCTGCACTGGGTAACGCCTCAGATGCTCTGTGTCCCTGTCA
           :::::::: :::::::: ::::::: :::::: :::::::::: :::::::::::: :
           TTCAAAAGAGAATCAGAGCCCTGCGCTGGGTTACGCCTCAGATGCTGTGTGTCCCTGTTA


           840        850        860        870        880        890
           ATGAGGAAATCCCTGAAGTGTCCGACATGGTGGTGAAAGCGATCACAGACATCATTGAGA
           :::: :: ::::: :::::::: :: ::::::::: :::::::::: :::::::: :
           ATGAAGACATCCCAGAAGTGTCTGATATGGTGGTGAAGGCGATCACAGATATCATTGAAA


           900        910        920        930        940        950
           TGGACTCAAAGCGTGTGCCTCGGGACAAGCTGGCCTGCATCACCAGGTGCAGCAAGCACA
           :::: :: :::::::::::::::: :::::::::::::::::::::: ::::::::::::::
           TGGATTCCAAGCGTGTGCCTCGAGACAAGCTGGCCTGCATCACCAAGTGCAGCAAGCACA
```


FIG. 11A

Murine Rin2/ Human HRIN2-2B1 clone (continued)


```
                440        450        460        470        480        490
Murine Rin2 960        970        980        990        1000       1010
        TCTTCAATGCCATCAAGATCACCAAGAATGAGCCAGCCTCTGCCGATGACTTCCTGCCCA
        ::::::.:::::::::::::::.:::.:::::::::: :: :::::: :::::.::::: ::::
        TCTTCNATGCCATCAAGATCNCCANGAATGAGCCGGCGTCTGCGGATGANTTCCTCCCCA
HRIN2-2B1

           1020       1030       1040       1050       1060       1070
        CCCTGATCTACATCGTCCTGAAGGGCAACCCCCCTCGCCTGCAGTCCAACATCCAGTACA
        :::: :::::: : :: ::::.::::: ::::::.: ::: :: :: :: ::::: :: :
        CCCTCATCTACTT-GTTTTGAANGGCA-CCCCCNC-CCTTCA-TCTAATATCCA-TATA

           1080       1090       1100       1110       1120       1130
        TCACTCGCTTCTGCAACCCCA-GCCGGCTCATGACGGGCGAGGATGGCTACTACTTCACC
        :::: :::::::: : :::: :::: :: :: :: :: .: ::.:::::::: ::: ::
        TCACGCGCTTCTGCCATCCCAAGCCGACTGAT-ACTGGANAAGANGGCTACTA-TTC-CC

           1140       1150       1160       1170       1180       1190
        AACCTGTGCTGTGCTGTGGCTTTCATTGAGAAATTAGACGCCCAGTCTTTGAATTTAAGT
         : ::::::::::::.::::::::::::::::: ::: :: :: :::..:::::::: ::: :
        CATCTGTGCTGTGCNGTGGCTTTCATTGAAAAACTAAAC-CCCNNTCTTTGAATCTAATT

           1200       1210       1220       1230       1240       1250
        CAGGAGGATTTTGACCGGTACATGTCTGGCCAGACATCCCCCAGGAAGCAGGAGTCTGAG
        :.::: :: ::::: :. ::::.: : :::::::.:: : ::.:: .::: : .: ::.:
        CNGGAAGAATTTGATCNCTACNTTTTTTGGCCNGA-ACCCNCCCNGAAACCANAAACTNAA

           1260       1270
        AGTTGGCCCCCGGAGGCCTGCTTAGG
        : ::::: :::::: :.:: ::::::::
        AATTGGTCCCCG-ANGCTTGCTTAGG
          790        800
```

FIG. 11B

```
            10        20        30        40
TNCCAANAANATATTCAAGTNTTTTTGGGGGGGGCNAAATT 40
CACCCCNTNCGGTTAAAGGAACCAATTGATNTTTTTTTAA 80
CAAAACCNGGGGAGTTTTTCCTTNCGGGGGGGGGTAAATANG 120
GGGNAACCCAAAGATTNTTGCNATTCAATGCACAGGNGGG 160
ATGTNAACTAAAAACGGAGTTAAATATTTTAGGGGGGGTTG 200
            210       220       230       240
ACAGCAACCTGCATNGTAGAACCTTTTTTTTNTTTCNGNG 240
GACNTTNTATAACNTAAATATACCATTGATGATTTTNTTC 280
CATTCAGTGACATCCACAGATTANGCAGCTATACTTGTGA 320
AATCGTGCATGAGGCCCCCAGGGCACCGTTTTAGAACAAC 360
GTCACTTCACACAGGCAGGTGAGAAAGGTTCTCTTGCTTT 400
            410       420       430       440
TCCAGTATCTTCCTAAGGATGGAGCCCAAAATTGCAGAGC 440
AGTAACTTTGGAATAAAACCAGGGTGGGTATAAAACTTCT 480
TATTCTTAAATTTACATATAAGATCTATTAAGCTTGACAC 520
ATCTGTGTCATCACGCACTGAAGACAGGAAGCAGTTCACT 560
GAGTCAGCTGGTTCCCAAGCTCGCACAGAAGGTGATAAGT 600
            610       620       630       640
TACTATCAAATGCCAGTGAGAATCTTCTTATAGAATAACC 640
TGGGCCCAAGTGATTTTAGTACAAAACTTGCCCTTCTTTG 680
GTTTAATTTTCTATGTGCTTTTAGGTGTGAATCCAGATAT 720
GCGGTCTTAATTCCTTTGGAAATACACAGTTCGTTTAGTT 760
ACTGTACACTCTGTTTGTTCAATAAACTGCATATCAACTT 800
            810       820       830       840
CCAAAAAAAAAAAAAAAAAAAAA 822
```

FIG. 11C
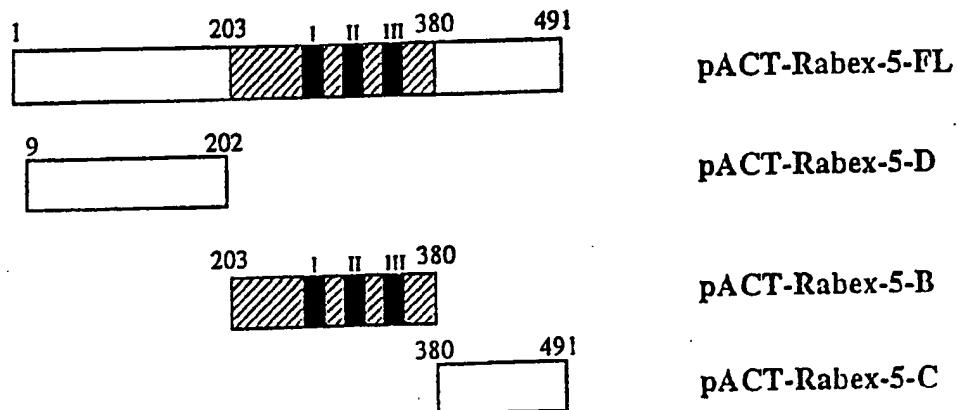
DNA-binding hybrid	Activation domain hybrid	Colony color	Relative β-Gal units
pAS2-mHRas	pACT-Rabex-5-FL	Blue	229
pAS2-mHRas	pACT-Rabex-5-D	Blue	14
pAS2-mHRas	pACT-Rabex-5-B	Blue	24
pAS2-mHRas	pACT-Rabex-5-C	Blue	138
pAS2-yRas2p	pACT-Rabex-5-FL	White	6
pAS2-yRas2p	pACT-Rabex-5-D	White	4
pAS2-yRas2p	pACT-Rabex-5-B	White	<1
pAS2-yRas2p	pACT-Rabex-5-C	White	<1

FIG. 12
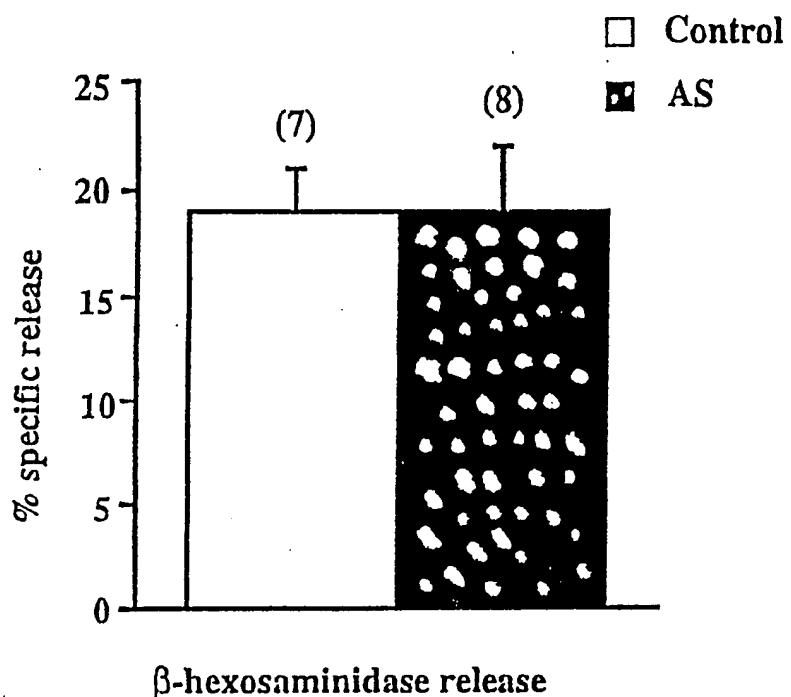
FIG. 13A
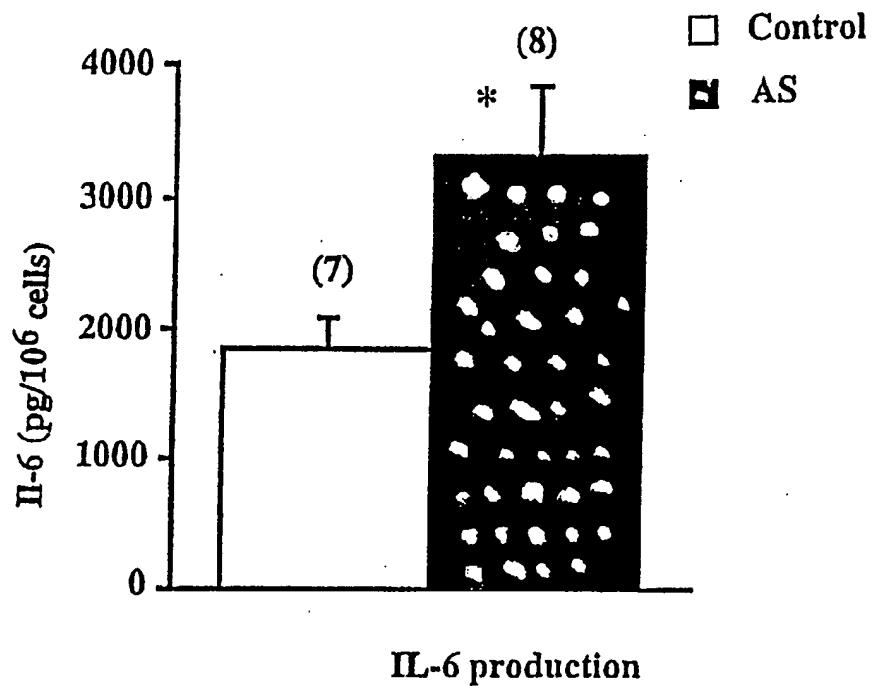
FIG.13B
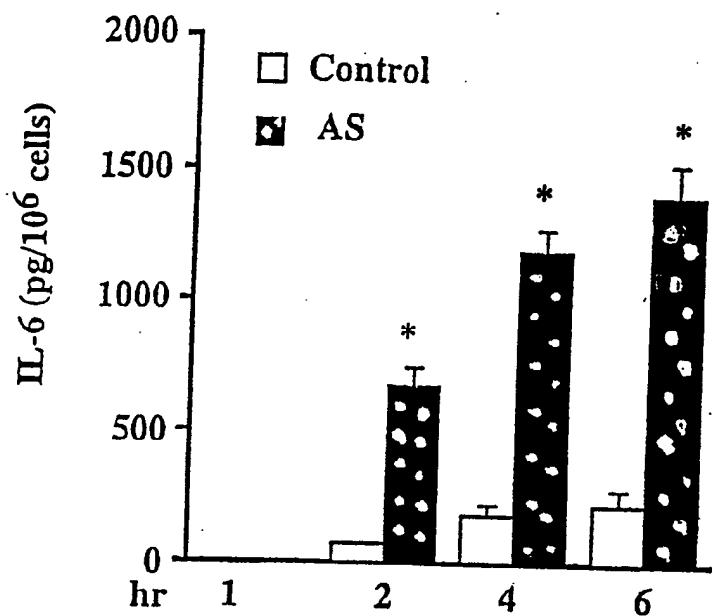
FIG. 13C
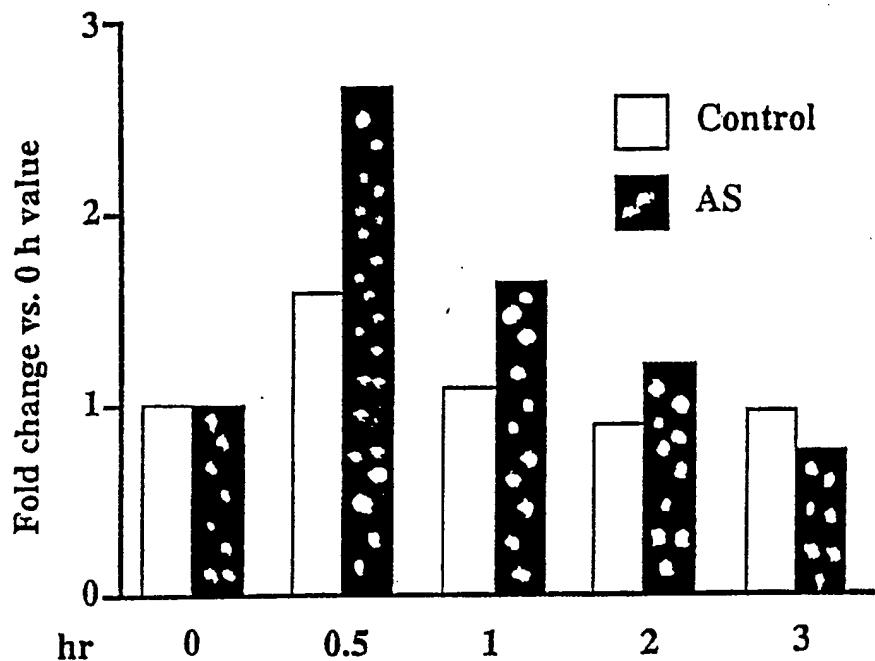
FIG. 14
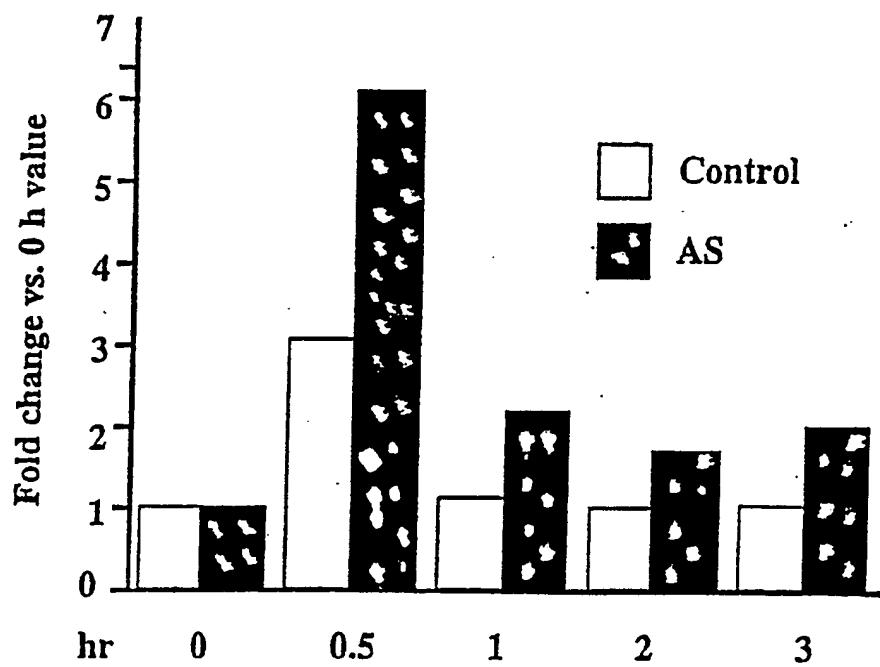
FIG. 15

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/12   C07K14/47   C07K16/18   C12Q1/68   G01N33/50
         G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   C12Q   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 666 314 A (COLD SPRING HARBOR LAB) 9 August 1995 SEQ ID NOS: 13-16 see page 12, line 53 - line 56 ---	9,10,12, 15,16
X	J. COLICELLI ET AL.: "Expression of three mammalian cDNAs that interfere with RAS function in Saccharomyces cerevisiae" PROC. NATL. ACAD. SCI., vol. 88, April 1991, pages 2913-2917, XP002087363 NATL. ACAD. SCI.,WASHINGTON,DC,US; see the whole document --- -/--	9,10,12, 15,16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 December 1998	28/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	HORNIG, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	L. HAN ET AL.: "Protein binding and signalling properties of RIN1 suggest a unique effector function" PROC. NATL. ACAD. SCI., vol. 94, May 1997, pages 4954-4959, XP002087364 NATL. ACAD. SCI.,WASHINGTON,DC,US; cited in the application see the whole document ---	9,10,12, 15,16
X	C.G. BURD ET AL. : "A yeast protein related to a mammalian ras-binding protein, Vps9p, is required for localization of vacuolar proteins" MOL. CELL. BIOL., vol. 16, no. 5, May 1996, pages 2369-2377, XP002087365 ASM WASHINGTON, DC,US cited in the application see the whole document ---	9,10,12, 15,16
A	H. STENMARK ET AL.: "Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic mmebrane fusion" CELL, vol. 83, 3 November 1995, pages 423-432, XP002087366 CELL PRESS,CAMBRIDGE,MA,US; see the whole document ---	1-23
P,X	HORIUCHI H ET AL: "A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function." CELL, (1997 SEP 19) 90 (6) 1149-59. JOURNAL CODE: CQ4. ISSN: 0092-8674., XP002087367 see the whole document -----	1-8,11, 13,14, 17-21

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INTERNATIONAL SEARCH REPORT

Box I Observations where ertain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 24-54
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

Please see Further Information sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.:   24-54

Claims 24 relating to agents which alter Rin2 activity relating to claims 25-57 could not be searched as the subject matters were not sufficiently disclosed.

Furthermore Claims 9,10,12,15,16, make no sense   SEQ ID NOS. 12,13,14,15 are not nucleotide sequences but amino acids

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0666314 A	09-08-1995	CA	2080920 A	21-10-1991
		EP	0537173 A	21-04-1993
		WO	9116457 A	31-10-1991
		US	5527896 A	18-06-1996